



**THE EFFECT OF PROCESS VARIABLES ON LIPID  
UTILISATION IN THE FERMENTATION OF  
*STREPTOMYCES CLAVULIGERUS***

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**Thesis submitted for the degree of Doctor of Philosophy from the  
University of London**

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## ABSTRACT

Many industrial fermentations use complex medium containing both carbohydrate and oils as their carbon sources. Oil supplements have been shown to increase the antibiotic titre and are a cheaper alternative carbon source, in terms of carbon per unit volume, when compared to carbohydrates. Oil supplements are also preferred on an energy supply basis and have natural antifoam properties. The major disadvantage to the use of oils in process media is the level of oil remaining at the end of the fermentation.

Research has been undertaken to examine the effects of process variables on lipid utilisation and residual oil levels in 5 litre batch fermentations of *Streptomyces clavuligerus*. The project illustrates the way in which both the chemical environment, media composition and method of pH control, and the physical environment, such as agitation rate and particle size, may influence growth, lipase activity, lipid utilisation and secondary metabolite production.

Changes in carbon source influenced growth and productivity. Addition of rapeseed oil increased final product titres by approximately 900%. The type and concentration of the nitrogen source was found to influence maximum lipase activity and initial oil utilisation rates. Media composition and pH control experiments had little effect on residual oil levels.

A gas chromatography method was developed in order to analyse the changes in the composition of oil during fermentation. Derivatisation of the fatty acids by methylation was successful and revealed that the composition of residual oil was similar to that of the initial rapeseed oil added. There was no indicated specificity in the utilisation of any individual fatty acids of rapeseed oil.

Increasing tip speed in the fermentation process from  $1.88 \text{ m s}^{-1}$  to  $2.83 \text{ m s}^{-1}$  increased maximum biomass levels, decreased lipase activity and did not affect clavulanic acid production. A tip speed of  $3.77 \text{ m s}^{-1}$  was detrimental to growth, increasing hyphal fragmentation, and decreased lipase activity. Increasing constant tip speed did not reduce residual oil levels. Antibiotic <sup>titres</sup> were relatively unaffected by changes in tip speed.

Addition of a surfactant to the complex medium, in order to facilitate oil droplet breakage and hence increase oil utilisation, was investigated. Addition of the surfactant decreased residual oil levels with an observed increase in final clavulanic acid titres.

This research has shown the effect of some chemical and physical parameters on lipid utilisation and clavulanic acid production in the 5 litre batch fermentation of *S. clavuligerus*. Lipid utilisation could not be enhanced by increasing lipase activity or varying media composition and agitation rates. Addition of surfactant to the process media significantly reduced residual oil levels. This approach may have potential as a generic method of reducing residual oil levels and enhancing secondary metabolite titres in industrial processes using lipid based medium.

## **ACKNOWLEDGEMENTS**

I would like to thank my supervisor, Dr. A. Ison, for his continued support and perseverance, without which I would not have been able to complete the project. Fermentations would not have been completed without the support of the pilot plant technical team, especially Mr Stuart Pope. Many thanks to all those in the Department of Biochemical Engineering for their help throughout my time at U.C.L. especially Dr Noushin Mirjalili for her assistance and friendship throughout her time at U.C.L. I would also like to thank Scott for his firm support, encouragent and constant belief in me.

I am particularly grateful to SmithKline Beecham Pharmaceuticals and my industrial supervisor, Dr. Dave Williams, for support and advice. I would like to thank Ruby and Sarah for their technical advice and support.

I would also like to acknowledge the support of the Biotechnology and Biological Sciences Research Council.

I would like to give special thanks to my advisor, Professor Malcolm Lilly, for his intellectual input and personal advice. He will be greatly missed.



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## NOMENCLATURE

A	Constant	( - )
a	Area per unit volume	m <sup>2</sup>
C	Capacitance	pF
C*	Oxygen concentration at gas/liquid interface	Kg m <sup>-3</sup>
C <sub>b</sub>	Constant	( - )
C <sub>G</sub>	Oxygen concentration in gas phase	Kg m <sup>-3</sup>
C <sub>L</sub>	Oxygen concentration in liquid phase	Kg m <sup>-3</sup>
D	Impeller diameter	m
d <sub>i</sub>	Impeller diameter	m
De	Effective oxygen diffusion coefficient	( - )
K	Constant	( - )
K <sub>L</sub>	Liquid phase mass transfer coefficient	( - )
K <sub>s</sub>	Constant	( - )
K <sub>r</sub>	Consistency index	( - )
m	Constant	( - )
N	Rate of impeller rotation	s <sup>-1</sup>
n	Flow behaviour index for power law fluids	( - )
P	Power consumption	W
P <sub>d</sub>	Power dissipation	W
P <sub>g</sub>	Gassed power	W
P <sub>ug</sub>	Ungassed power	W
Re	Reynolds number	( - )
Re <sub>c</sub>	Critical Reynolds number	( - )
Sp	Spreading coefficient	( - )
t <sub>c</sub>	Circulation time	s <sup>-1</sup>
V	Volume	m <sup>3</sup>
V <sub>L</sub>	Liquid volume	m <sup>3</sup>
V <sub>s</sub>	Superficial gas velocity	m s <sup>-1</sup>
α	Constant	( - )
β	Constant	( - )
χ	Constant	( - )
ε	Rate of turbulent energy dissipation per unit mass	W kg <sup>-1</sup>
γ	Shear rate	s <sup>-1</sup>
η <sub>a</sub>	Viscosity	Pa s
λ	Kolmogorov	m
μ	Viscosity	Pa s
μ <sub>App</sub>	Apparent viscosity	Pa s
v	Fluid linear velocity	m s <sup>-1</sup>
ν	Kinematic viscosity	m <sup>2</sup> s <sup>-1</sup>
ρ	Fluid density	Kg m <sup>-3</sup>
σ <sub>wg</sub>	Interfacial tension between water and gas phase	N m <sup>-1</sup>
σ <sub>og</sub>	Interfacial tension between oil and gas phase	N m <sup>-1</sup>
σ <sub>ow</sub>	Interfacial tension between oil and water phase	N m <sup>-1</sup>
τ	Shear stress	Pa
τ <sub>0</sub>	Yield stress	Pa



## **1. INTRODUCTION**

### **1.1. Project introduction**

This research was carried out in order to investigate the effects of both physical and chemical pilot plant scale process parameters on lipid utilisation in a complex process media containing a partial lipid carbon source. A literature review was initially undertaken to investigate the importance of actinomycetes and streptomycetes in terms of classification and morphology, secondary metabolite production and their economic significance. *Streptomyces clavuligerus* produces many classes of antibiotics including the commercially important clavulanic acid which was discovered through screening for  $\beta$ -lactamase inhibiting compounds. The review of present literature contains information regarding the background behind clavulanic acid discovery and biosynthesis, information regarding the patented fermentation process and the present market for clavulanic acid.

Many antibiotic processes use mixed substrate carbon sources, including lipids, in order to increase product titres. A review of present research involved in the use of mixed carbon substrates within Streptomycete fermentations concluded many advantages to the use of lipids including a notable increase in antibiotic titre and the additional advantage of natural antifoam properties of oils. However, it was discovered that there are many economic disadvantages to the use of a lipid substrate in a batch process. It was reported that over a third of the initial substrate remained unused at the end of fermentation. Metabolism of a lipid substrate also requires a higher oxygen demand. The residual oil problem has obvious economic significance when considering the cost of raw materials, downstream product isolation and antibiotic purification.

Most microorganisms produce a lipase enzyme to metabolise the triglycerides present in the oil carbon source to their constituent fatty acids and glycerol. Increasing the activity of the lipase, and investigating any specificity to certain components of the initial oil feed, may be the key to reducing the residual oil levels. The research review therefore includes an investigation into the effect of the physical and chemical environment on lipase activity and enhanced production of a lipase enzyme in order to increase oil metabolism.

The effect of process variables within a fermentation vessel includes a wide variety of physical and chemical parameters. Current research is presented in which methods of reducing the residual oil problem in both batch and fed-batch fermentation have been investigated. The rheology of antibiotic producing fermentations was considered to be significant due to the effect of oil addition on rheology and the mixing environment in relation to Streptomyces fermentation and lipid utilisation. A review on present research into the effects of mixing and shear on oil uptake and organism morphology and productivity was also investigated. The concentration of dissolved oxygen and carbon dioxide within the fermenter environment was also studied in order to obtain information on the effects on morphology, productivity and oxygen transfer in a variety of filamentous fermentations and applying what is already known to investigate lipid utilisation and the link with clavulanic acid production in *S. clavuligerus*.

## **1.2. Research aims and objectives**

Despite the amount of literature reviewed indicating a link between lipid utilisation and increased product titres, details on the environment within a fermentation vessel, with respect to enhancing lipid utilisation, remain unclear. If a more strategic approach is to be taken in the improvement of yields, through increased lipid metabolism, then both physical and chemical parameters must be studied. The aim of this research was to investigate the effects of fermentation parameters on lipid utilisation in *Streptomyces clavuligerus* on a 5 L pilot plant scale. Lipid metabolism, lipase activity and clavulanic acid production were monitored from a complex production media. A detailed investigation of the process parameters will yield information that will generate a greater understanding of the processes involved in lipid metabolism. Increased understanding may lead to developments of methods to increase product titres and decrease downstream processing costs through reduction in residual oil levels. The knowledge gained will have global applicability to the many antibiotic producing processes that use lipids as co-substrates in their production medium.

## **2. LITERATURE REVIEW**

### **2.1. Actinomycetes**

#### **2.1.1. Importance and morphology**

Existence of actinomycetes as a distinct group, actinomycetales, has been recognised for many years (Buchman, 1917). Actinomycetes are Gram positive, sporulating, saprophytic bacteria (Goodfellow *et al.*, 1988) with elongated cells or filaments that usually show some degree of branching. Despite the diverse morphology of the actinomycetes they can be placed into two groups: nocardioform or sporoactinomycetes. Nocardioform actinomycetes form hyphae, which will fragment into rod-like or coccoid shaped elements to generate new mycelia. Greater extremes of morphology are seen within the sporoactinomycetes, but all have the ability to form spores. Asexual, thin walled spores, called conidia or conidiospores, develop from septal formation in or on defined parts of the hyphae. Most spores are not particularly heat resistant but can withstand considerable desiccation. The organism germinates from these spores to form germ tubes which in turn give rise to a network of hyphae. Mycelial growth is related to their ability to break down insoluble organic materials for intake by the organism.

All organisms within this complex group are defined as chemoorganotrophs, requiring some form of organic carbon source, inorganic nitrogen source, mineral salts and an approximate neutral pH in order to grow (Gusek and Kinsella, 1992). Most actinomycetes are not motile; when motility is present it is confined to flagellated spores. Although the actinomycetes have been the subject of extensive literature, many aspects of their physiology and roles within the natural environment have still to be understood.

Morphology of the actinomycete plays a crucial role in achieving a successful antibiotic production process, to be referred to as fermentation herein. Many factors influence the morphology of actinomycetes such as species, strain, growth media or their physical environment. Therefore actinomycetes exhibit structures which range from simple rods and cocci to complex mycelial organisation (Goodfellow *et al.*, 1983). Structures within the fermentation process range from compact pellets to fluffy mycelia.

It is assumed that filamentous actinomycetes elongate through apical extension by deposition of wall polymers only at the very tip of the hyphae (Kretschmer, 1982). However, autoradiographic studies seemed to indicate wall synthesis was not restricted to hyphal tip as preferential incorporation of N-acetyl-D-[1-<sup>3</sup>H] glucosamine label, a precursor of peptidoglycan, took place over a broad segment of the hyphae and not only at the tip (Gray *et al.*, 1990). Labelling in regions away from the hyphal tip may be due to wall synthesis, turnover and thickening.

Actinomycetes also change morphologically during a process, due to changes within their environment, from cell growth and maintenance, primary metabolism, to secondary metabolism, which results in the production of bioactive metabolites. It is these compounds that are of great industrial significance, although their function within the organism remains unclear (Doull and Vining, 1991).

The outstanding property of actinomycetes is their ability to produce a variety of bioactive compounds including antibiotics, vitamins, enzymes, immuno-suppressors and immuno-modifiers (Piret and Demain, 1983), herbicides, insecticides and antiparasitic compounds (Berdy, 1988). Thousands of secondary metabolite producing actinomycetes have been isolated and have found practical application in human and veterinary medicine reinforcing their economic and industrial importance (Bushell, 1983). For this reason actinomycetes attract a great deal of scientific attention.

The second world war brought intensive antibiotic research with one of the most important discoveries being the production of antibiotics by the actinomycetes. The first purified antibiotic was actinomycin, isolated by Waksman and Woodruff in 1940 from *Streptomyces antibioticus*. This antibiotic was found to inhibit the activity of both Gram positive and Gram negative organisms. Actinomycin was later superseded by the first antitubercular antibiotic, streptomycin, discovered by Schatz (1944), which proved to be more effective against a wider range of pathogens. These events, and the earlier discovery of penicillin from *Penicillium notatum* by Fleming in 1928, marked the beginning of the antibiotic era and diverted a great deal of scientific effort towards the search for novel compounds.

### 2.1.2. Streptomycetes

Streptomycetes are members of the family Streptomycetaceae and are considered to be the most common members of the order actinomycetales (Buchman, 1917). Streptomycetes are soil dwelling sporoactinomycetes that are highly aerobic. During growth on solid media their morphology is complex, forming two types of mycelia, aerial and substrate mycelia, which are morphologically and physiologically different (Kalakoutsii and Agre, 1976). The aerial hyphae, which are the less highly branched, bear chains of spores enclosed in a thin sheath which are their reproductive units (Williams and Wellington, 1983; Dietz, 1983). The form of aerial mycelium is influenced by factors such as media composition, incubation temperature and nutrient depletion. The sporulation process is also influenced by growth conditions which may result in variation in morphology and physiology of the organism. Sporulation is rarely observed in submerged culture although it has been shown that exhaustion of media components, such as phosphates, may induce sporulation (Kalakoutski and Agre, 1976). The highly branched substrate mycelia develop through apical extension at the hyphal tip in order to penetrate and solubilise organic debris by the secretion of extracellular enzymes. Branching of the hyphae may result from a localised depletion in nutrients in or around the branch point. Hyphae contain cross walls which are important when considering age and morphology of the organism since they should reduce fragmentation and decrease the loss of cell contents during submerged fermentation. This growth pattern of Streptomycetes is similar to the growth and differentiation of a fungal fermentation.

The classification of Streptomycetes is being continuously revised due to the application and development of isolation techniques. The presence of LL-diaminopimelic acid, (LL-DAP), has been used as the main characteristic between Streptomycetes and other Actinomycetes. Within the group Streptomycetes, pigment production, spore formation patterns and the presence of volatile compounds producing distinctive smells, such as geosmin, are used in identification procedures. There is a problem of over classification within the Streptomycete group due to the intense screening of thousands of species for novel bioactive compounds by the pharmaceutical industry. Therefore, in the early 1960's the International Streptomycetes Project, (ISP), began in order to standardise methods for characterisation and to provide reliable descriptions of all new species.

Streptomycetes are now considered the most successful source of antibiotics and other secondary metabolites. The most productive of the Streptomycetes is *Streptomyces* spp. which account for approximately 93% of actinomycete bioactive substances, (Bushell, 1988). Closely related Streptomycetes often produce variants on the same class of antibiotic. Examples include two structurally and functionally similar compounds, chlortetracycline and oxytetracycline, which are produced by *Streptomyces aureofaciens* and *S. rimosus* respectively. However, condicidin, streptomycin and cyclohexamide, which differ structurally but share similar biosynthetic pathways, are all produced by *S. griseus*. Other Streptomycetes produce related compounds such as *S. clavuligerus* which secretes  $\beta$ -lactam compounds such as penicillin N, clavams including clavulanic acid, and also produces a group of cephalosporins.

### 2.1.3. *Streptomyces clavuligerus*

*S. clavuligerus* was first discovered by Waksman and Henrici in 1943 but not named as a new species until 1971 by Higgins and Kastner when its main bioactive compounds were discovered. *S. clavuligerus* is an aerobic Gram positive sporoactinomycete with aerial mycelium bearing spores at some time in its life cycle. The organism tends to grow slowly as branching filaments which segment during growth and produce club shaped cells. *S. clavuligerus* shows mainly mycelial growth and rarely exhibits the more compact pellet form in submerged cultures. Among actinomycetes, *S. clavuligerus* is unusual in its ability to assimilate only a narrow range of carbon sources such as starch, and other simple sugars, i.e. glycerol, yet lacks the ability to assimilate glucose (Aharonowitz and Demain, 1978). This is thought to be due to an inadequate mechanism for the uptake and conversion of the glucose to a glycolytic intermediate (Vining *et al.*, 1987).

*S. clavuligerus* produces structurally related antibiotics with the same mode of action, i.e. inhibiting cell wall synthesis. It is reported that the organism produces five  $\beta$ -lactam type antibiotics in total, clavulanic acid, deaceloxycephalosporin, penicillin N, cephalosporin C and the O-carbonyl derivative of deacetylcephalosporin (Hu *et al.*, 1984).

## 2.2. Clavulanic acid

In order to counter growing resistance to  $\beta$ -lactam antibiotics two main approaches are available. One approach is to discover a  $\beta$ -lactam antibiotic inherently stable towards  $\beta$ -lactamase enzymes and the other is to find an inhibitor of the  $\beta$ -lactamase activity. In 1967 a programme of research began at SmithKline Beecham in which soil organisms were screened for the possible production of naturally occurring  $\beta$ -lactamase inhibitors. A family of  $\beta$ -lactamase inhibitors, olivanic acids, were first discovered in *Streptomyces*, and further work lead to the discovery of clavulanic acid first detected in *Streptomyces clavuligerus* (Brown *et al.*, 1976).

### 2.2.1. Clavulanic acid structure and biosynthesis

Clavulanic acid is a fused bicyclic  $\beta$ -lactam (Howarth *et al.*, 1976), structurally different from penicillins and cephalosporins with an oxygen replacing the usual sulphur within the  $\beta$ -lactam ring at the carbon in position 4, Figure 2.1. The origins of clavulanic acid have been extensively reviewed by Romero *et al.* (1988) and Reading and Cole (1977).

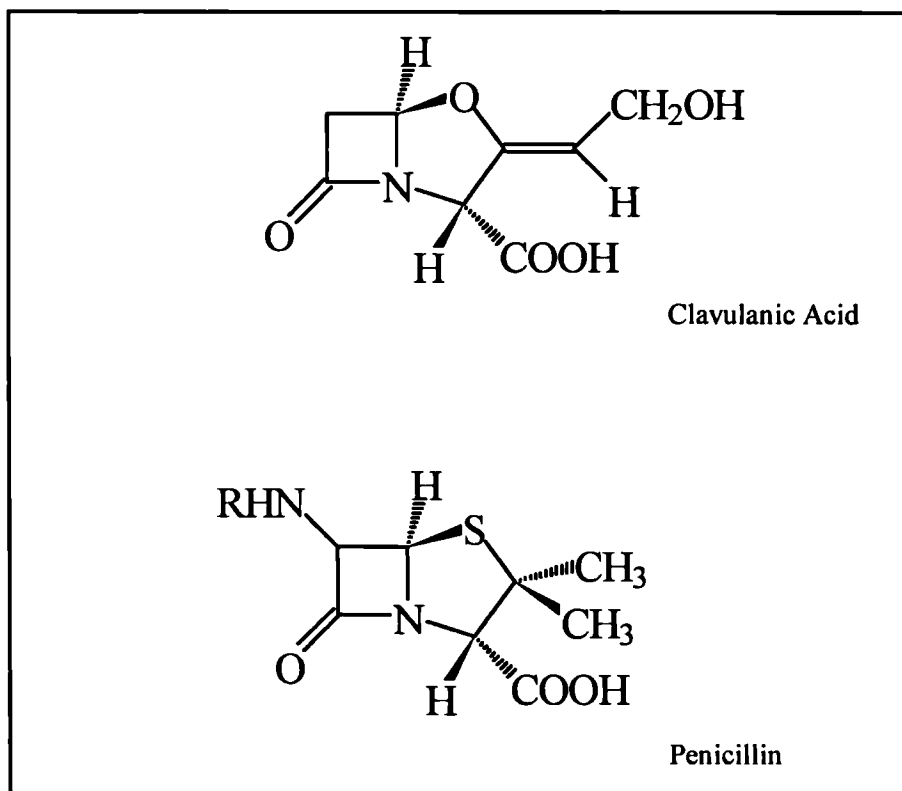


Figure 2.1. Clavulanic acid molecule and a general penicillin structure.

*Streptomyces clavuligerus* is an unusual organism in that it produces two major groups of  $\beta$ -lactam antibiotics. The first group includes the bicyclic rings containing sulphur, the penicillins, cephalosporins and cephamycins and the second group the oxygen-containing ring systems of clavulanic acid. Quite distinct biochemical pathways exist in *Streptomyces clavuligerus* for the principle  $\beta$ -lactam antibiotic families (Jensen and Westlake, 1988). The genes for clavulanic acid biosynthesis are located downstream of the relatively well distinguished cephamycin cluster in *S. clavuligerus* (Ward and Hodgson, 1993) but there is still limited information about the clavulanic acid cluster (Aidoo *et al.*, 1994; Hodgson *et al.*, 1995).

Studies on the origins of clavulanic acid over the last couple of decades have revealed an interesting biosynthetic pathway. Pioneering studies carried out by Elson (Elson and Oliver, 1978; Stirling and Elson, 1979; Elson, 1981; Elson *et al.*, 1982) on the incorporation of radiolabelled precursors concluded that acetate enters the clavulanic acid molecule via the tricarboxylic acid (TCA) cycle from which 2-oxoglutarate provides the 5 carbon skeleton. Research has shown that both arginine and ornithine are the precursors for clavulanic acid biosynthesis. Through amino acid labelled studies and the presence of an arginase enzyme which converts arginine to ornithine (Romero *et al.*, 1986) it was thought that arginine is converted to ornithine and that this C5 amino acid is the direct precursor of clavulanic acid. Evidence that arginine and not ornithine is the amino acid taken into the clavulanic acid biosynthetic pathway was obtained by the SmithKline Beecham group (Valentine *et al.*, 1993) by blocking enzymes in the urea cycle of *S. clavuligerus* through UV irradiation.

Research by Townsend and Ho (1985) and Pitlik and Townsend (1997) showed that glycerol provides the 3 carbon skeleton of the  $\beta$ -lactam ring. A scheme of biosynthesis of clavulanic acid is shown in Figure 2.2. Feeding experiments using labelled precursors have demonstrated that glycerol and pyruvate (Pitlik and Townsend, 1997), propionate (Elson and Oliver, 1978), and D- and L-glycerates (Townsend and Ho, 1985) are specifically incorporated into the  $\beta$ -lactam ring. The most recent report using optically active  $^{13}\text{C}$  and  $^2\text{H}$  labelled lactates and glycerates implies that pyruvate and not glycerate is the primary metabolic precursor of the three  $\beta$ -lactam carbons (Thirkettle *et al.*, 1998).



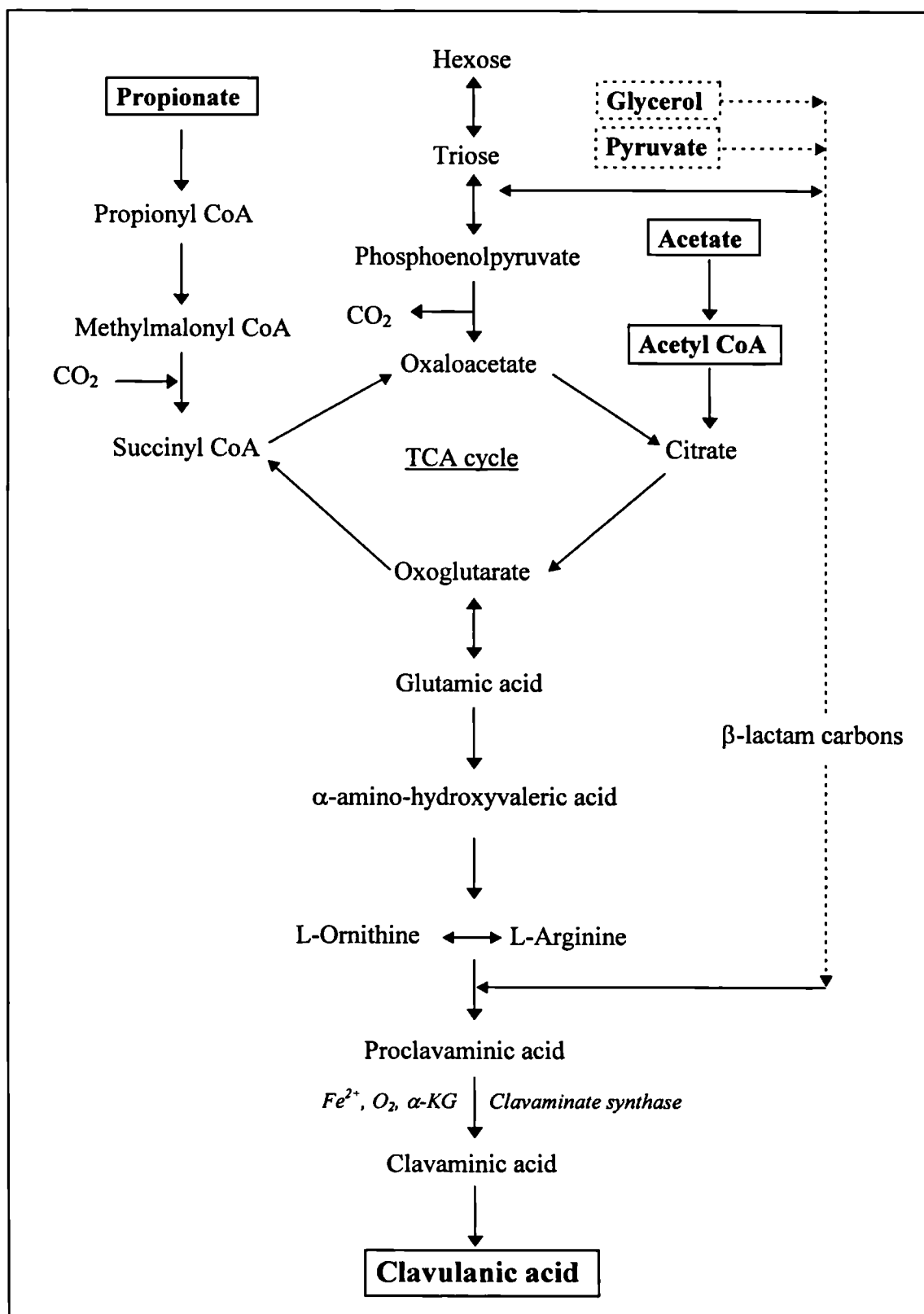


Figure 2.2. Clavulanic acid biosynthesis pathway.

Almost all knowledge of the biosynthetic pathway up to this point had been deduced by feeding radio labelled precursors which had to undergo an unknown number of enzymic reactions to yield clavulanic acid. In order to obtain information regarding the biosynthetic intermediates between the proposed primary metabolic precursors and clavulanic acid cell free studies have been carried out (Elson *et al.*, 1988). It was proposed that the C5 and C3 precursors form proclavaminic acid. The reaction involving the transformation of proclavaminic acid to clavaminic acid requires the enzyme clavamate synthase, (CS), an  $\alpha$ -ketoglutarate dependent oxygenase central to the biosynthesis of clavulanic acid. It has been reported that iron dependent enzymes play a central role in the construction of the bicyclic structure of these molecules and are also essential to their antibiotic activities (Marsh *et al.*, 1992). Baggaley *et al.* (1997) have recently published a comprehensive review of the chemistry and research leading to the discovery of the biosynthetic pathway of clavulanic acid and other clavams.

### 2.2.2. The function of clavulanic acid

Clavulanic acid is a potent progressive inhibitor of certain  $\beta$ -lactamases (Baily, 1984) and especially the clinically important group of plasmid-mediated  $\beta$ -lactamases produced by *Enterobacteriaceae*, *Haemophilus influenzae* and *Neisseria gonorrhoeae* and the chromosomally-mediated  $\beta$ -lactamases from *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, and *Bacteroides fragilis* (Baggaley *et al.*, 1997). Clavulanic acid itself has only weak, though broad-spectrum antimicrobial activity against most bacteria, with a minimum inhibitory concentration of 25-125  $\mu\text{g mL}^{-1}$  (Reading and Cole, 1977). Its antibacterial mode of action is *via* inhibition of the penicillin binding protein.  $\beta$ -lactamase inactivate penicillins, cephalosporins and related antibiotics and was first recognised by Abraham and Chain (1940) in a strain of *Escherichia coli*. It was later established as the mechanism of resistance in penicillin resistant strains of *Staphylococcus aureus* (Kirby, 1944). Resistance to  $\beta$ -lactam antibiotics increased until in the early 1960's a frequency of 70-80% resistance was not uncommon in hospitals in many countries of the world (Rolinson, 1994).  $\beta$ -lactam inhibition has also been reported in *Haemophilus influenzae*, *Enterococcus faecalis*, *Moraxella catarrhalis*, *Neisseria meningitidis* (Rolinson, 1994).

The discovery of clavulanic acid has dramatically opened the way for clinically valuable synergism with  $\beta$ -lactamase susceptible agents such as ampicillin and amoxycillin

(Franklin and Snow, 1985). An example of which is Augmentin™, manufactured by SmithKline Beecham Pharmaceuticals, first introduced into the U.K. in 1981 (Rolinson, 1994). The microbiology of amoxycillin/ clavulanate has been extensively reviewed over the fifteen period, 1978-1993, by Rolinson (1994). Clavulanic acid has recently attained generic status and is, therefore, of widespread interest.

### 2.2.3 The fermentation process

Due to the economic significance of clavulanic acid the full details of the process involved in the fermentation of *S. clavuligerus* are not fully disclosed. Pilot plant scale procedures for clavulanic acid production from *S. clavuligerus* have been described in British patent No. 1,563,103, and other producing Streptomycetes in Japanese patents 53,104,796 and 55,162,993. All patents show that the most important nutrients with respect to clavulanic acid production are soyabean protein, a high carbon sugar source and addition of rapeseed oil.

The complex nature of carbon metabolism in *S. clavuligerus* is not well understood. The use of glucose as a sole carbon source will decrease antibiotic production when compared to starch or dextrans (Romero *et al.*, 1984). This may be due to rapid metabolism as carbon catabolites have been reported to inhibit penicillin biosynthesis (Revilla *et al.*, 1984) and cephalosporin production (Aharonowitz and Demain, 1978) by *Penicillium chrysogenum* and *S. clavuligerus* respectively. Salowe *et al.* (1990) reported that yields of clavulanic acid were quite variable and concluded that a complex soybean and starch medium was generally superior to a defined glycerol/sucrose/proline/glutamate media described by Romero *et al.* (1984). The addition of a lipid as an alternative carbon source is important as a means of avoiding carbon catabolite repression due to its low solubility in the fermentation media (Butterworth, 1984) and has been shown to increase many antibiotic titres, Section 2.4.3.

Clavulanic acid has been reported to be one of several  $\beta$ -lactam compounds produced by *S. clavuligerus* in response to changes in available nutrients (Romero *et al.*, 1984). The level of phosphate in the fermentation media has also been shown to be important in antibiotic synthesis with respect to changing carbohydrate metabolism, stimulating primary metabolism and inhibition of precursors (Martin and Demain, 1980). The effect

of phosphate on clavulanic acid synthesis showed that at the level of 10-100mM the phosphate dramatically reduced clavulanic acid titres (Romero *et al.*, 1984). Lebrihi *et al.*, (1987) reported that phosphate levels control clavulanic acid by repression of the clavulanic acid synthetase enzymes.

From pilot plant scale information that is available, the process involves the use of stirred, aerated vessels as described in British patent number 1,571,888. The inoculum source is usually a mycelial or spore suspension, the mycelial being preferred. A temperature range of 26-30°C and a pH of between 6.0 and 7.5 are the identified culture conditions. pH is a major economic concern in an industrial process in terms of optimising productivity as clavulanic acid itself is only stable between a pH of 6.5 and 7.2 (Tarbuck *et al.*, 1985). Media contains 0.1-1.0% complex organic or chemically defined sources of nitrogen, 0.1-5.0% carbon source, mineral salts and trace elements (Cole *et al.*, 1978).

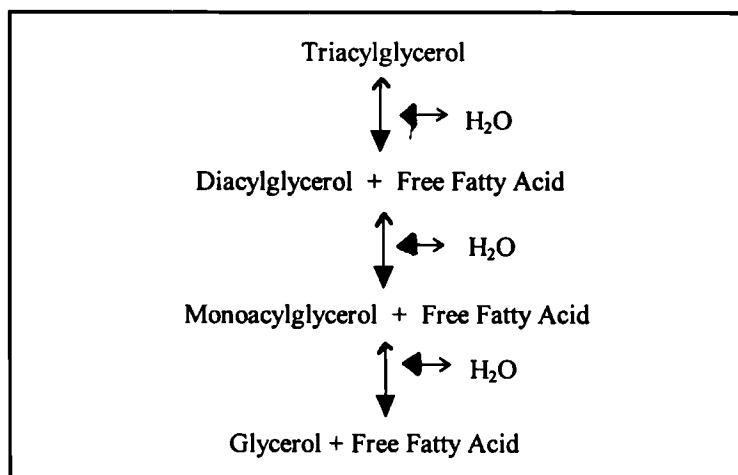
## **2.3. Lipase**

### **2.3.1. Functions of a lipase**

A lipase is an enzyme that catalyses the hydrolysis of fats and oils to their constituent glycerols and free fatty acids at a rate dependent on the specificity of the lipase involved. Figure 2.3. shows the reaction involving the lipase enzyme. Lipases are characterised by their ability to catalyse the hydrolysis of ester bonds at the interface between the insoluble substrate and the aqueous phase in which the enzyme is soluble, (Marangoni, 1994). Thus lipases catalyse the hydrolysis of a wide range of insoluble fatty acid esters although triglycerides are normally the preferred substrates. Hydrolysis of water soluble carboxylic acid esters by true lipases is very slow.

Specificity of lipase is controlled by the molecular properties of the enzyme, the structure of the substrate and factors affecting binding of the enzyme to the substrate (Jensen *et al.*, 1983). Specificity is a comparative difference in rates of catalysis of certain reactions and there are many different types of lipase specificity. These have been identified as specific to the substrate, to the position of the fatty acid, specific to the type of fatty acid, or they may be stereospecific. Lipase may also be specific to any combination of these. Lipase biosynthesis occurs in animals, plants (Sonnet and

Antonian, 1988) and microorganisms. Among the latter, moulds are widely recognised as the best lipase source (Hatzinikolaou *et al.*, 1996).



**Figure 2.3. Lipase reaction**

### 2.3.2. Microbial lipase

Screening and isolation of microorganisms for lipase activity from soil samples or other biological materials is relatively easy and most frequently carried out employing agar plates containing triglycerides. Lipase catalysed hydrolysis gives rise to clear zoning or precipitation of fatty acid salts. A comprehensive account of the sources from which lipase can be isolated is given by Shelly *et al.* (1987).

One of the first observations of microbial lipolytic activity was made by Alford *et al.* (1964). Since, there has been widespread discovery of microorganisms producing lipase. Literature contains information on the lipolytic activity of a wide range of microorganisms ranging from yeast (Montesinos *et al.*, 1996) to fungi, such as *Aspergillus niger* (Hatzinkolaou *et al.*, 1996), *Penicillium citrinum* (Szatzer and Maliszewka, 1989) and *Rhizopus oligosporus* (Nahas 1988) and many bacteria such as *Pseudomonas aeruginosa* (Khor *et al.*, 1986) and some *Streptomyces* spp. (Borman *et al.*, 1993) such as *Streptomyces lactis* (Lukoseva *et al.*, 1982) and *S. cinnamomeus* (Sommer *et al.*, 1997). The majority of the known microbial lipases tend to be acidic proteins with a molecular weight of 20 - 60,000 Daltons and have been characterised with respect to their pH activity profiles, stability and activity at elevated temperatures, positional specificity of tri-glyceride hydrolysis and fatty acid specificity (Godtfredsen, 1990).

Commercially microbial lipases are very important and depending on the enzymes' specificity have found various applications in the dairy industry (Stead, 1986), the manufacture of detergents (Fujii *et al.*, 1986), oleochemistry, cosmetics, pharmaceuticals and in the treatment of domestic wastes. This has led to lipase becoming a valuable biotechnological tool and industry now has the ability to produce the enzyme on a large scale. Their significance has led to a greater understanding of the catalytic activity of lipase and the enzyme kinetics required in order to improve process economics. The industrial applications of lipase have been reviewed by Macrae (1983).

The majority of microorganisms secrete extracellular lipase, some microorganisms contain the lipase inside the cell and in other organisms the enzyme may be surface associated or cell bound. Extracellular lipase is the most commonly studied due to its industrial significance and many examples are given in the literature: *Aspergillus niger* (Hatzinikolaou *et al.*, 1996), *Penicillium cyclopum* (Iwai *et al.*, 1975), *Geotrichum candidum* (Jacobsen *et al.*, 1989) *Rhizopus oligosporus* (Nahas 1988) and *Candida rugosa* (Veeraragan, 1989). A review of the production of exogenous lipases by bacteria, fungi and actinomycetes is reported by Sztajer *et al.* (1988). Fewer examples of intracellular and membrane bound lipase enzymes are reported (Novotny and Dolezalova, 1993; Gobetti *et al.*, 1996; Misset *et al.*, 1994; Jacobsen *et al.*, 1989).

### 2.3.3. Lipase from *Streptomyces*

Lipase activity must be present in order to utilise the oil in streptomyces fermentations in which a lipid co-substrate is used to increase secondary metabolites. Lipase activity has been detected in a small number of streptomyces. Sztajer *et al.* (1988) found lipolytic activity in *Streptomyces* spp. PCM27 and PCM33 and *S. fradiae* using olive oil and tributyrin substrate reagents. Rapp and Backhaus (1992) showed that *S. caelestis*, *S. lavendulae* and *S. lipmani* exhibited extracellular lipase activity towards a triolein substrate. Borman *et al.*, (1993) tested 243 strains of *Streptomyces* for lipase activity against plate tests using tributyrin agar. 51% showed significant lipolytic activity.

### 2.3.4. Effect of the fermenter environment on lipase activity

Extracellular lipase and membrane bound or cell associated enzymes are in constant contact with the surrounding media. Therefore the effect of the chemical and physical environment surrounding the cell such as pH, metal ion concentration, shear forces, oxygen tension, surfactants and other media components affect lipase activity, production and stability. Growth conditions may influence the properties of the enzyme produced as well as a ratio of extracellular to intracellular enzyme (Taipia *et al.*, 1992).

#### 2.3.4.1 Media components

Lipase activity has been shown to be affected by the physical state of the media. Rivera-Munoz *et al.* (1991) found that, using *Penicillium candidum*, lipase activity was higher in submerged culture than on solid media but the actual production of the lipase occurred earlier and tended to be more stable on the solid medium. The concentration and form of nitrogen source present in the media has been found to affect lipase activity. The use of peptone in the growth media has been reported to increase the lipolytic activity in *Pseudomonas aeruginosa* and *Ps. fluorescens* (Cutchins *et al.*, 1952). Results indicated that the activity of the enzyme increased as the complexity of the nitrogen source decreased. Cordenons *et al.* (1996) concluded that lipase expression improved 2-3 fold by applying changes in the nitrogen source in the fermentation medium. Minimal levels of lipid such as butter oil, lard oil, olive oil and certain fatty acids have been reported to be required for both extracellular (Suzuki, 1988), and cell bound (Novotny and Doletalova, 1993) lipase activity. The addition of carbon sources such as starch and sucrose in combination with certain nitrogen and phosphorous sources have been shown to increase lipase activity in *Aspergillus niger* (Pokorny *et al.*, 1994) and *Penicillium citrinum* (Sztajer and Maliszewska, 1989). Recent research by Hatzinkolaou *et al.* (1996) reported that it was possible to substantially enhance the lipase activity of *A. niger* by optimising the interaction between carbon and nitrogen sources. Four carbon sources, glucose, sucrose, corn oil and olive oil, and three nitrogen sources, ammonium phosphate, ammonium sulphate and peptone were applied to a 4x3 experimental design. The results concluded that the combination of peptone and corn oil yield highest lipase activity.

Anionic surfactants such as Tweens have been shown to enhance lipase activity in a number of different organisms (Nahas, 1988). Jacobsen *et al.* (1989) also concluded

that by adding olive oil or Tween 80 to a basic media extracellular and cell bound lipase yields and activity could be enhanced in cultures of *Geotrichum candidum*. Maximum yields were found with the addition of Tween 80 alone, which resulted in a six-fold increase in extracellular lipase activity when compared to the basic media. During the earlier stages of growth in medium containing olive oil the proportion of cell-bound activity was higher than that of extracellular activity, and a delay in the secretion of extracellular lipase was found.

High concentrations of oils have been shown to have an inhibitory effect on detected lipase activity (Gilbert *et al.*, 1991). This may be due to the toxicity of the fatty acids released from lipase activity. Lipase formation has been reported to be suppressed by the presence of mono and disaccharides or glycerol within a media (Macrae, 1983). Iron, magnesium and calcium are also reported to have some inhibitory effects on enzyme activity. SDS and copper ions have been shown to completely inhibit lipase activity (Maliszewka and Mastalerz, 1992).

#### 2.3.4.2 Physical environment

The influence of the physical environment within a fermentation also affects the production and activity of a lipase enzyme. It is well known that the rate of hydrolysis of oil by a non-oil soluble lipase is a direct function of the surface area of the oil-water interface (Marangoni, 1994). Good mixing is therefore important in lipase catalysed reactions and this is usually achieved by mixing the molten or solvent dissolved oil with lipase in the aqueous phase in a stirred tank reactor, (Lee and Choo, 1989). Lee and Choo (1989) investigated the effects of shear on inactivation of extracellular lipase production by *Candida cylindracea*. They concluded that free lipase is sensitive to denaturation by an increase in shear rate <sup>and</sup> ~~but not~~ shear stress and that lipase activity is affected by the length of time the enzyme is exposed to the shear forces.

Jacobsen *et al.* (1989) found that increased lipolytic activity of extracellular and cell-bound lipase from *G. candidum* was observed in a 1 L fermenter when compared with a 300 mL shake flask. Results of work carried out by Tahoun *et al.* (1982), on *G. candidum* concluded that shaking or aeration in shake flask experiments resulted in lower concentrations of lipase in the culture liquid. Marek and Bednarski (1996) reported that lipase activity increased when lowering the working volume of the media



contained within a fermenter. It seems that aeration of the culture in shake flasks and dissolved oxygen tension within a fermentation are critical to lipase production and activity.

Research by Cavanagh (1996) concluded that the lipase enzyme produced by *S. clavuligerus* used to hydrolyse the rapeseed oil in the fermentation media appears not be extracellular but associated with the cell membrane. It was also concluded that the activity of this particular lipase was affected by changes in the pH and the temperature at which the culture was held, by phosphate and nitrogen levels, as well as the presence or absence of oil in the media. At higher oil levels there was greater lipase activity which suggested that, for the lipase associated with *S. clavuligerus*, a critical oil level was necessary for expression. No lipase activity was detected in the absence of an oil carbon substrate.

From all the studies and reports noted above it is clear that careful manipulation of the physical and chemical parameters within a process are extremely important for maximum lipase activity.

## **2.4. Lipids**

### **2.4.1. Introduction to fats and oils**

The terms fats and oils are essentially interchangeable, those which are liquid at ambient temperature are called oils and those that are solid or semi-solid are fats. The physical and chemical characteristics of individual fats and oils are determined mainly by their fatty acid composition. A fatty acid is a compound synthesised in nature via condensation of malonyl coenzyme A units by a fatty acid synthase complex. The fatty acid usually contains an even number of carbon atoms, although odd numbers do occur, and branched chains may be produced from an appropriate precursor. The fatty acid may be saturated, containing only carbon-carbon single bonds, unsaturated, contain double bonds or may contain substituent groups, incorporated through the appropriate enzymes. There are universal symbols which may be used to describe the structure of a fatty acid. The symbol 18:0 denotes a fatty acid containing 18 carbon atoms and no double bonds, 18:2 denotes an 18 carbon fatty acid containing two double bonds.

The simplest, most abundant lipids are the triacylglycerols, triglycerides, which are condensation products of 1 molecule of glycerol with 3 molecules of fatty acid. Nearly all industrially important fats and oils of animal and plant origin consist almost exclusively of triglycerides. Di-glycerides and mono-glycerides contain 2 and 1 mole of fatty acid per mole of glycerol respectively and are usually found in trace amounts unless the oil or fat has undergone partial hydrolysis. These are important molecules found as intermediates in the biosynthesis of lipids, as intracellular messengers and in the regulation of vital processes in mammals. Commercially, monoglycerides and diglycerides are an important class of food emulsifiers and are used in the manufacture of many foodstuffs.

The ability to utilise fatty acids, and the oils and fats which contain them, is found widely amongst bacteria, yeast and moulds. The ester link between the glycerol backbone and the fatty acid is hydrolysed by a lipase enzyme, Section 2.3. The glycerol then enters the Embden-Meyerhof pathway and is converted to a glycolytic intermediate later in the oxidation cycle. The fatty acids released are extremely toxic to the cell and therefore must be utilised efficiently into their coenzymeA thioesters. The thioester is activated for degradation of the fatty acyl chain by the  $\beta$ -oxidation cycle, Figure 2.4. The fatty acyl-CoA ester continues the oxidative cycle and the cycle is repeated until the fatty acid molecule is completely broken down to two carbon unit molecules. At each turn of the cycle a molecule containing two carbon units, acetyl-CoA, is released. If the fatty acid entering the oxidative cycle has an odd number of carbon units then the final product from the cycle is a propionyl-CoA molecule. It is the acetyl-CoA that may itself be a starter unit for the precursors in the formation of many antibiotics. Alternatively, as the acetyl-CoA is being produced from  $\beta$ -oxidation, the pyruvate, which will normally produce acetyl-CoA if fatty acids are not present, may be used for the synthesis of precursors to the antibiotic biosynthesis.

#### **2.4.2. Commercial oils**

A wide variety of different oils and fats are used on an industrial scale. The majority of the fats and oils used are plant derived although some animal fats, such as lard, may be favoured due to quicker metabolism as they are unsaturated (Stowell, 1987). The most widely produced oil is soyabean oil, whose plant seeds have a 19-20% oil content. Problems have arisen from the use of soyabean oil due to its high linoleic acid content,

5-18%, which causes poor flavour stability and unpleasant odours. Palm oil is also of high commercial value on a large scale although the seed has an oil content below 20%.

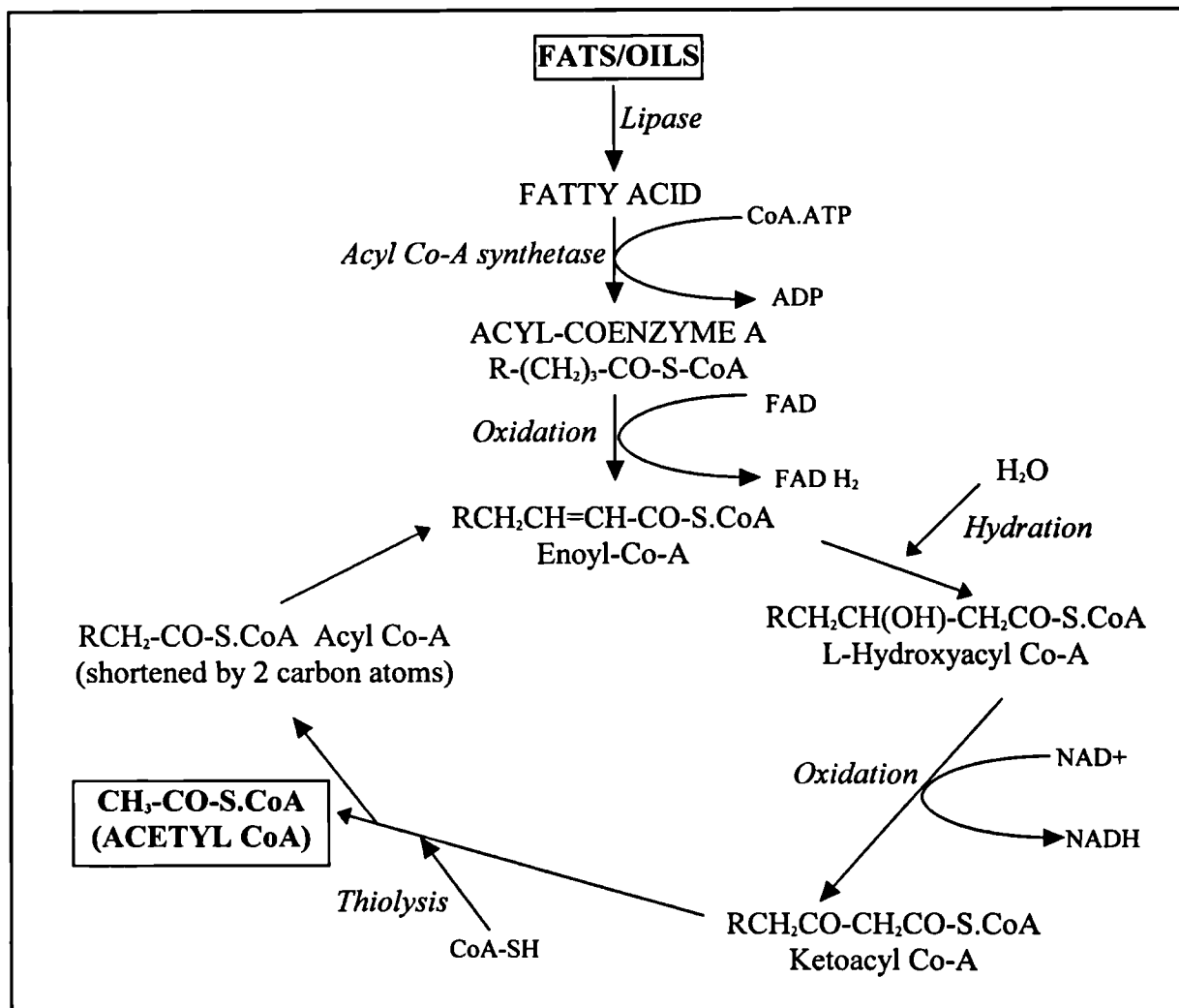


Figure 2.4. β-oxidation cycle. (Stryer, 1988).

For the purpose of this project the most important oil is rapeseed oil (RSO) also known as calza or canbra oil. The oil is obtained from the seeds of the rape and turnip rape plants and is a major oil-seed crop of Canada, Europe and other northern climates (Florkowski and Purcell, 1989). In general, there are three types of RSO produced which vary in their fatty acid composition each type being used for either edible or industrial purposes dependent on the percentage of erucic acid content. The three types are high erucic acid (HEAR) low erucic acid (LEAR) and zero erucic acid which contain 55-25%, 5-0%, and 0% erucic acid respectively. RSO also contains high levels of unsaturated fatty acids and a very low percentage of fatty acids of chain length shorter than C16. The type of plant and the production of the oil are dependent on the climate

and plants have now been manipulated via selection and breeding programs to reduce the harmful erucic acid and sulphur content. Currently planted rapeseed cultivars are free of erucic acid. The improvement in propagation techniques means that new cultivars are expected to result in higher yields with increased resistance to disease. As the adoption of these new techniques spread the price of rapeseed oil is expected to decrease therefore encouraging wider use (Florkowski and Purcell, 1989).

### **2.4.3. Oil as a substrate**

The choice of a carbon substrate for a fermentation process is highly complex requiring both technical and economic input. Glucose is the usual carbon and energy source for antibiotic-producing microorganisms. However, glucose is rapidly catabolised and this may decrease the rate of biosynthesis due to catabolite repression of the biosynthetic enzymes involved in production. This problem may be alleviated by the control-rate feeding of glucose which is a technique employed in the commercial production of penicillin. Other methods to reduce catabolite repression include the use of complex sugars, such as starch, or alternative carbon sources with low solubility in process media such as oils (Pan *et al.*, 1959).

#### **2.4.3.1. Advantages of an oil based carbon source**

Bader *et al.* (1984) describes some of the technical points favouring the use of oil compared to carbohydrate as a carbon source. Energetically a typical oil contains approximately 2.4 times the energy of glucose on a weight basis (Stowell, 1987). This is an important consideration when looking at commodity prices for a large scale process. Oil is also preferred on a volume basis when compared to carbohydrate (Stowell, 1987). For example, it takes 1.24 litres of soyabean oil to add 10 kcal of energy to a fermentation process, whereas, it takes greater than 5 litres of glucose or sucrose to add the same amount of energy, assuming the sugars are added as 50% w/w solutions.

Addition of oils to a fermentation medium will reduce surface tension at the liquid-gas interface in the fermenter. Therefore oils were first used in an antibiotic process as carriers for antifoams. The antifoam property of oils is another advantage when compared to carbohydrates. This is an important consideration if the recovery process

involves methods such as ultrafiltration as many proprietary antifoams may cause problems with membrane fouling.

The main advantage to the use of oil co-substrates within a process media is the increase in secondary metabolite titre noted by much research, Table 2.1. Initial studies by Stefanick *et al.* (1946) report that the increase in titre was brought about by the oil being used as an antifoam agent. This was disproved as the addition of any non-oil based antifoam should bring about the same result under this theory. Hattman (1945) reported an increase in antibiotic titre when *Penicillium notatum* was grown on solid state medium containing corn oil as the major carbon source. This also disproves the theory of Stefanick in that the use of an antifoam is not necessary in solid state medium. Pan *et al.* (1959) supported the theory that the use of fatty oils in a corn steep-CaCO<sub>3</sub> medium accelerate penicillin production in *Penicillium chrysogenum*. With the correct ratio of mineral oil to fatty oil, such as corn oil, the fermentation could proceed at a rate 50% higher and last 1-2 days longer than a lactose control.

Tan and Gill (1984, 1985a, 1985b, 1987) used olive oil, lard and mutton tallow supplemented media for batch fermentations of *Saccharomyces lipolytica* and *Pseudomonas fluorescens*. The presence of lipid enhanced growth of the organisms when compared to growth on a carbohydrate providing that they were mechanically dispersed as evenly as possible throughout the fermentation media. Rezanka *et al.* (1984) reported that the addition of 2-4% oils to synthetic media used for the cultivation of *Streptomyces cinnamonesin* increased the production of monesin. Tan and Ho (1991) found that, when using palm oil and its fractions as the main carbon source, the growth and production of penicillin by *Penicillium chrysogenum* appeared to be strain dependent. They concluded that this may be due to poor transport of the fatty acid inside the cells which, in turn, may lead to poor induction of lipase production and therefore a decrease in the formation of the precursor acetyl-CoA. They also found that 2% crude palm oil could replace 5% lactose as the main carbon source for good growth and penicillin production and could also help produce a stable pH.

Park (1994) investigated vegetable oils to act as the sole carbon source for production of the antibiotic, cephamycin C, both in shake flask and batch fermentation. Cephamycin C was shown to be exclusively produced even when soybean oil was used

as a sole carbon source. The product yield from the soybean oil was 4.7 times higher than that of starch media.

**Table 2.1. Lipid supplements which enhance antibiotic production.**

Antibiotic	Producing Organism	Lipid supplement enhancing production	Reference
Fungichromin	<i>Streptomyces cinnamomeus</i>	Oleic acid	McCarthy <i>et al.</i> (1955)
Sistomycosin	<i>Streptomyces viridosporus</i>	Soybean meal	Ehrlich <i>et al.</i> (1955)
Filipin	<i>Streptomyces filipensis</i>	Palmitic acid	Brock, (1956)
Lagosin	<i>Streptomyces cinnamomeus</i>	Palm oil	Bessel <i>et al.</i> (1961)
Aureofungin	<i>Streptomyces cinnamomeus</i> var. <i>terricola</i>	Soybean meal	Thirumalacher <i>et al.</i> (1964)
DJ 400 series DJ400B <sub>1</sub> DJ400B <sub>2</sub>	<i>Streptomyces surinam</i>	Olive oil, Coconut oil	Siewert and Kieslich (1971)
Neomycin	<i>Streptomyces fradiae</i>	Oleic acid, Palmitic acid	Okazaki <i>et al.</i> (1974)
Monesin	<i>Streptomyces cinnamomensis</i>	Rape seed oil	Rezanka <i>et al.</i> (1984)
Cephameycin C	<i>Streptomyces</i> sp. 6221	Soyabean oil	Park <i>et al.</i> (1994)
Neomycin	<i>Streptomyces fradiae</i>	Soyabean oil	Ohta <i>et al.</i> (1995)
Rapamycin	<i>Streptomyces hydroscopicus</i>	Soyabean oil	Box <i>et al.</i> (1995)
Tylosin	<i>Streptomyces fradiae</i>	Rapeseed oil,	Choi <i>et al.</i> (1996)
Tylosin	<i>Streptomyces fradiae</i>	Palm oil, Palmitic acid	Lee <i>et al.</i> (1997)
Avermectin	<i>Streptomyces avermitilis</i>	Lard oil	British patent 572056
Clavulanic acid	<i>Streptomyces clavuligerus</i>	Rape seed oil	British patent 1563103

More recent evidence includes investigations into efficient tylosin production from *Streptomyces fradiae* using rapeseed oil (Choi *et al.*, 1996) and palm oil supplemented media (Lee *et al.*, 1997). Vegetable oils were compared to starch and glucose as alternative carbon sources. Rapeseed oil and palm oil enhanced tylosin production. Palm oil and its fractions have been found to enhance both clavulanic acid and cephamycin C production when compared to sucrose and glycerol supplemented media in *S. clavuligerus* fermentations (Lee and Ho, 1996).

#### 2.4.3.2. Disadvantages of an oil based carbon source

Although there are many advantages to the use of lipids as a carbon source within a process, disadvantages include problems which arise due to a residual oil level that is higher than that of carbohydrates, which often approximates to zero (Stowell, 1987). This may be due to a physical limitation of oil mass transfer in the fermenter, the inability of the organism to metabolise certain components of the input oil material, or it may be due to physical inaccessibility of the oil to the organism. A high level of residual oil is undesirable as it may increase viscosity and therefore possibly reduce oxygen transfer efficiency in the fermenter. The increase in viscosity of the process medium can also lead to the entrapment of air into the broth which may cause considerable foaming problems and difficulties in downstream processing. The residual oil may however have negligible viscosity effect relative to a non-Newtonian nature of Streptomycete fermentations. However, the residual oil level will interfere with product recovery consequently increasing production costs.

In addition to the residual oil problem lipid is less well oxidised than carbohydrate and therefore for a given energy input into a fermentation the oil has approximately 7% higher oxygen demand. This is an important consideration for many antibiotic processes where oxygen maybe a limiting substrate. The effects of oils on mass transfer within the fermenter environment is described in section 2.5.4.

#### 2.4.3.3. Approaches to solving the residual oil problem

The problem of residual oil may be due to many factors including physical limitation of oil mass transfer in the process, inability to metabolise certain components of the lipid substrate or physical inaccessibility of the oil to the organism. Due to the economic advantages to the use of an oil substrate, reduction of the residual oil in a antibiotic process has been under intense investigation.

As the residual oil problem may be due to incomplete metabolism of the lipid substrate investigations have been made into the influence of fatty acids on growth and productivity. *Cephalosporium acremonium* has been cultivated in fermentation medium containing fatty acid methyl esters as carbon sources for cephalosporin production (Sohn *et al.*, 1994). Results concluded that the methyl ester of oleic acid was utilised much quicker than the linoleic acid methyl ester. Cellular fatty acid content of cells

varied throughout the fermentation and was dependent on the medium used. In the methyl oleate medium cell morphology changed from mainly hyphae to arthrospores and conidia. In contrast, in a sucrose medium the cells remained hyphal at the end of the fermentation.

*Sporobolomyces odoratus*, which produces secondary metabolites used in food flavourings, was grown in broth to which fatty acids and oils were added (Lee and Chou, 1994). The fatty acids decanoic and dodecanoic acid reduced growth and productivity when added to the culture. Whereas castor oil, if added 24 hours into the fermentation, enhanced both growth and productivity. The fatty acids contained in castor oil, palmitic, stearic, oleic, linoleic, and ricinolenic acids, were further added separately to the fermentation (Lin *et al.*, 1996). Only linoleic and ricinolenic acids partially inhibited growth, the other fatty acids had no significant affect. The level of linoleic acid did not decrease throughout the fermentation indicating that the organism did not metabolise this fatty acid.

*S. roseosporus* produces complex lipopeptide antibiotics which contain a common cyclic peptide and different fatty acyl side chains. By employing carbon limited fed-batch techniques, fatty acids of length C8 to C12 were successfully employed as precursors for the biosynthesis of the antibiotic (Huber *et al.*, 1990a). The efficiency of incorporation into the antibiotic was related to the length of lipid precursor, with C10 and C12 fatty acids being the preferred substrates. The effect of addition of short chain fatty acids on spiramycin, a macrolide antibiotic produced by *Streptomyces ambofaciens*, has been investigated by Khaoua *et al.* (1990). Some short chain fatty acids are catabolic products of amino acids, such as valine, and can be incorporated into the carbon skeleton of some antibiotics. The addition of short chain fatty acids stimulated the production of spiramycin when grown on medium containing dextrans and ammonium chloride. The results from addition of short chain fatty acids to the medium suggest that fatty acids from primary metabolism would be assimilated in secondary metabolism according to their nature.

More recently, Lee *et al.* (1997) has investigated tylosin production by *S. fradiae* on palm oil, palm kernel oil, and their fractions as well as fatty acids and glycerol at 1% w/v as main carbon sources in shake flask cultures. Of all the carbon sources tested all



supported cell growth except for oleic acid. Both palm oil and palm kernel oil and their fractions were comparable to glucose in terms of tylosin production. The most significant result is that the long chain fatty acids resulted in increased tylosin production in addition to supporting good bacterial growth, especially palmitic acid. Industrially this result is very important as it offers the prospect of directly using palmitic and stearic acids which are readily available from oleochemical plants. This procedure will avoid the non-utilisation of oleic with palm oil as the feed stock.

Lee and Ho (1996) investigated the growth and clavulanic acid production in *S. clavuligerus* in a palm oil medium and its fractions. *S. clavuligerus* grew well on palm oil, palm kernel oil, and its fractions and glycerol, but showed no growth on oleic or lauric acid. Palm olein was the best carbon source for clavulanic acid production, though all the palm oil fractions produced a considerable amount. When palmitic or stearic acids were used little clavulanic acid was produced.

Increasing agitation rates in a fermentation process causes a shift to a smaller oil particle size since higher turbulence intensity is more effective in the breaking of droplets (Chatzi *et al.*, 1991). Increasing agitation has to be balanced with the increase in shear affecting growth and productivity. Choi *et al.* (1996) investigated the effect of increased agitation rates on the residual rapeseed oil levels on a 1.5 litre scale batch fermentation. They concluded that increasing the mechanical agitation will reduce the size of the oil droplets in the fermentation medium thus increasing the specific area of the oil and the oil consumption increased with increased surface area.

Due to the impractical applications of increasing agitation rates on an industrial scale Mitsuru *et al.* (1996) include a mineral support mesh in the fermentation of *Streptomyces* spp. In the absence of the support the oil formed large droplets around the hyphae. When the support was added to the culture the oil droplets were shown to concentrate around the mesh which reduced oil droplet sizes. The mineral support used in this research both immobilised the bacteria and improved the dispersion of the oil droplets. The improved oil consumption improved yields and increasing dispersion will again increase interfacial surface area between oil and mycelia when compared to fermentations minus the support and results in a lower residual oil level.

Addition of an emulsifier to the lipid supplemented media may reduce the size of the oil droplet therefore increase surface area to volume ratios and may increase oil consumption. The presence of a low concentration of surfactants has been reported to both stimulate and inhibit microbial growth and/or product formation (Sukan *et al.*, 1989). Addition of surfactants will affect downstream processing costs since the residual oil and surfactant must be removed from the product. Another approach to reducing oil droplet size is to increase the media temperature (Chatzi *et al.*, 1991). This is not a practical solution as increased temperatures will also affect the growth and therefore production from the organism.

Physical breakage of the oil droplet to emulsify the oil may also enhance oil utilisation and will eliminate downstream processing costs due to removal of emulsifying agents. Shiomori *et al.* (1995) prepared a polydispersed emulsion system by homogenisation and a monodispersed emulsion using a silica-porous-glass (SPG) membrane emulsification method. Rates of olive oil hydrolysis were affected by the concentration of olive oil and lipase, the interfacial area and emulsion droplets diameter. It was concluded that the rate limiting step of lipid hydrolysis was desorption of the product from the interface.

## **2.5. Rheology of filamentous fermentations**

### **2.5.1. The nature of fermentation broths**

Viscosity is the most significant property affecting the flow behaviour of a fermentation broth. This behaviour will have an affect on pumping, mixing, heat and mass transfer and aeration within the fermentation process. This in turn influences the course of the fermentation, response and reliability of sensors and the downstream recovery processes. Therefore, the viscosity contributes significantly to both process design and economics.

Low viscosity media are easy to handle and mass and heat transfer rates are usually high enough to ensure that microorganisms can grow at a sufficient specific growth rate. The highly filamentous nature of the Streptomycete group influences the rheological nature of the fermentation with respect to biomass concentration, growth rate and morphology. The increase in fluid viscosity can also result from a high substrate concentration,

secretion of highly viscous products and interactions between substrates and cell morphology and products and cell morphology (Charles, 1978).

Mixing problems are created by highly viscous fermentation suspensions. The increase in viscosity increases mixing time within the broth and may lead to the creation of stagnant zones. These zones will in turn lead to instability within the fermentation with respect to control systems due to time lags and concentration gradients of both products from, and media nutrients to, the organism. Heat transfer, mixing time and cooling of the process are also made more difficult by the variation in viscosity throughout the fermenter.

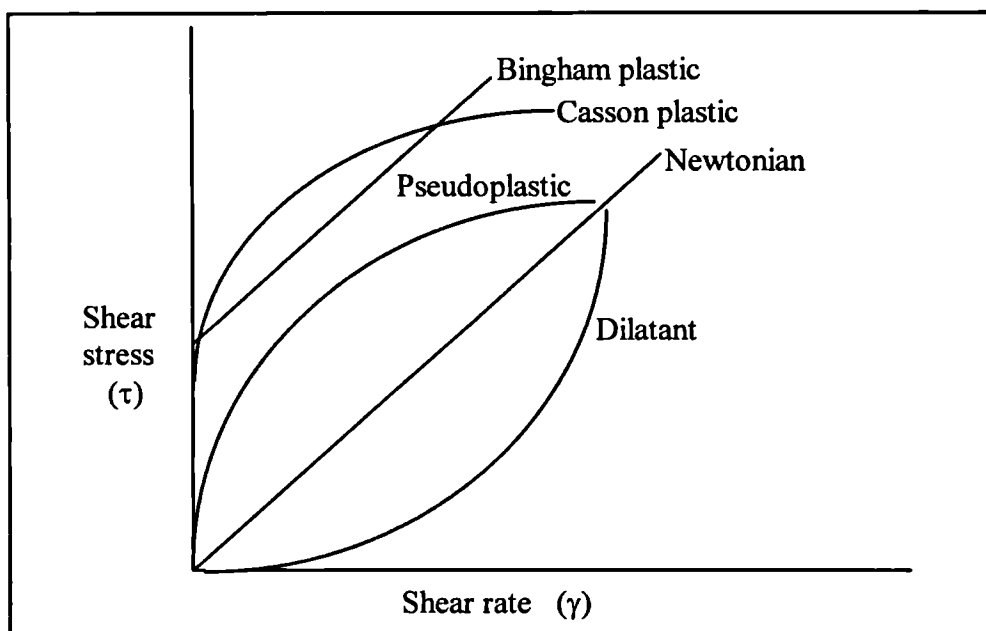
### 2.5.2. Rheological models

Mathematical models of rheological behaviour represent idealisation of actual material behaviour. Sometimes these models are extremely accurate over a range of shear rates; at other times, especially in microbial systems, the idealisation is an approximation of real behaviour but is nevertheless useful in providing predictions on the effect of shear on a material.

It is well established that the heterogeneous broth generated in industrial filamentous fermentations are non-Newtonian leading to relatively low viscosity in regions of high shear rate, near the impeller, and very high viscosities in regions of low shear, near the wall (van Suijdam and Metz, 1981). The Power law model (Tuffile and Pinho, 1970) is frequently used to describe the rheological behaviour of Streptomyces fermentation broths. This law defines the behaviour of a fluid that increases in viscosity with an increase in shear rate ( $\dot{\gamma}$ ), but does not include a yield stress ( $\tau_o$ ), the value of shear stress ( $\tau$ ), below which there is no flow, and can be used for both Newtonian and non-Newtonian fluids;

$$\tau = \tau_o + K (\dot{\gamma})^n \quad (2.1)$$

A summary of rheological parameters is given in Figure 2.5.



**Figure 2.5. Correlation of rheological parameters**

Rheology is dependent on any number of factors such as concentration and morphology of the organism and is affected by operating parameters of the fermentation. It is reported that *Streptomyces* exhibit definite rheological changes throughout the submerged fermentation process (Demnerova *et al.*, 1984) and it is therefore unclear which non-Newtonian parameters best describe a *Streptomyces* fermentation.

### 2.5.3. Rheological effects on mass transfer

The critical operation in an aerobic fermentation is often the supply of oxygen to the cells. The solubility of oxygen in water is very low and therefore the culture may be easily starved. In highly viscous fluids transfer of oxygen from gas to liquid phase is limited and therefore mixing and mixing times are critical. The greatest oxygen transfer rate therefore takes place near the impeller region where shear rates and power dissipation are highest.

A correlation between oxygen transfer rates and rheology of filamentous fermentation broth has been estimated using  $k_L a$ . One of the first studies to connect rheology and oxygen transfer rates was carried out by Deindorfer and Gaden (1955). These experiments concluded that there was an observed 85% decrease in  $k_L a$  as the viscosity of a fermentation broth of *Penicillium chrysogenum* increased. In general, a decrease in  $k_L a$  is observed as the viscosity of the broth increases (Gbewonyo, 1992).

There have been many studies to determine methods for measurement and evaluation of oxygen transfer coefficients in fermentations since oxygen transfer rates determine production capacities. An equation for the calculation of  $k_L a$  of non-Newtonian fluids was formulated by Ryu and Humphrey (1972);

$$k_L a = K (P/V)^\alpha (V_s)^\beta (\eta a)^x \quad (2.2.)$$

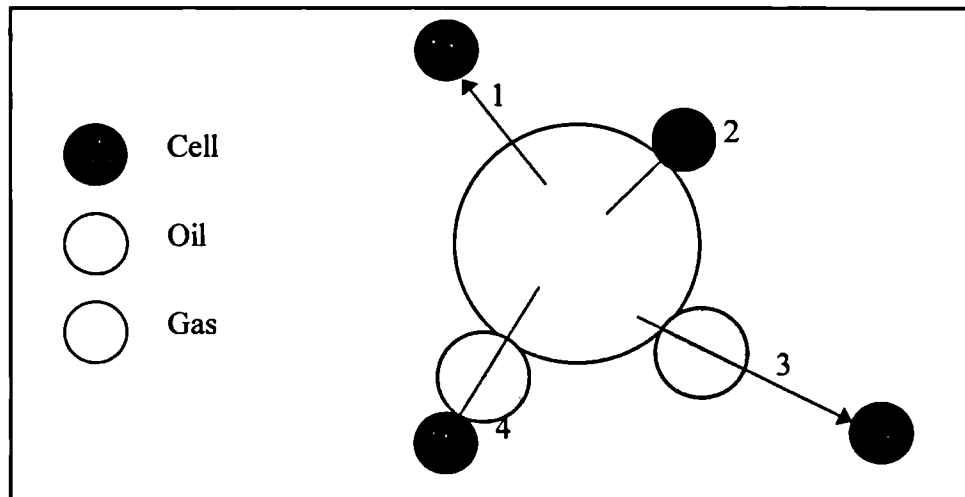
Equation 2.2. is highly scale dependent and does not incorporate measurements of mixing time and therefore is not totally accurate. Methods used to model the activities of viscous broth within a process involve homogeneous polymers and are therefore not accurate for the non-Newtonian microbial fermentation. Therefore morphology of an organism is a major determinant in flow calculations and the accurate determination of actual effects of the broth on mass transfer and rheology differ due to variations in techniques and properties encountered during measurements caused by the nature of the broth itself. These measurements would be further complicated by addition of media components such as oils.

#### **2.5.4. Effect of oil on rheology and mass transfer**

Oxygen solubility is very low in an aqueous fermentation media and limitations occur when oxygen demand exceeds supply. This causes the dissolved oxygen level in the aqueous phase to fall below the critical level required for a particular process. Various methods have been investigated in order to increase the oxygen supply to the organisms. Methods include the use of immobilised preparations (Enfors and Mattiasson, 1983), chemically generating oxygen by using a hydrogen peroxide/catalase system into the aqueous phase (Ibrahim and Schegel, 1980) and the use of oxygen vectors which have a higher oxygen solubility than water and supply oxygen to the media and surrounding cells (Rols and Goma, 1989).

The growth of an organism in a fermentation containing oils, such as rape seed oil, creates a four phase system. This system consists of a continuous phase, containing the soluble media components, and three dispersed phases which include the organism and insoluble medium components, solid phase, the air bubbles, gas phase, and the oil, liquid phase. The dispersed phases are maintained within a turbulent environment within the fermentation and this promotes constant renewal of gas-oil interfaces. The oil phase may influence mass transfer by either absorbing the oxygen, transferring the

oxygen to other phases or by influencing the fluid mechanics of the continuous phase. The various ways that oxygen can flow to reach the bacterial cells are shown in Figure 2.6.



**Figure 2.6. Possible ways of oxygen transfer from bubbles to cells.**

**(Rols and Goma, 1989).**

#### 1) Gas-liquid-solid transfer:

In the absence of oxygen-vectors the oxygen molecules are dissolved in the aqueous phase and used in the fermentation media by the organisms.

#### 2) Gas-solid transfer:

The organism may use oxygen directly at the interface between it and the gas bubble. Some organisms tend to crowd the surface of the gas bubble at the gas-liquid interface and therefore the concentration of the cells in this area is higher than in the bulk liquid. The cells themselves will therefore affect and enhance oxygen transfer rates.

#### 3) Gas-oil-liquid-solid transfer:

The oil may dissolve the oxygen which can then be transferred to the aqueous phase, the fermentation media, where it is utilised by the organism. From estimated values of the volumetric transfer coefficient of oxygen transfer through the water film surrounding the gas bubble and of overall oxygen transfer from water to oil droplet it has been concluded that the main resistance to oxygen absorption into the oil-water emulsion exists in the film around the gas bubbles (Yoshida *et al.*, 1970; Yamane and Yoshida,

1974). Oxygen transfer from oil to water is much faster than from air to oil and therefore it is thought that direct oxygen transfer from air to water is accomplished.

Further studies by Rols *et al.* (1990) proposed that the oil phase is an active intermediary for oxygen transfer from gas to water suggesting that the oxygen transfer rate may increase by layering of oil as a thin film at the gas-liquid interface. The spreading coefficient,  $Sp$ , for an oil droplet on a water surface is defined as:

$$Sp = \sigma_{wg} - (\sigma_{og} + \sigma_{ow}) \quad (2.3.)$$

When  $Sp$  is greater than or equal to zero the oil will spread on the surface of the water and if  $Sp$  is less than zero the oil will form droplets. The  $Sp$  value is dependent on the oil used.

Roques *et al.* (1987) proposed a model explaining the interaction between air bubble-oil droplet in water in a study of water-hydrocarbon separations by flotation. For a spreading coefficient of greater than zero the system goes through a recovery of the bubble by the droplet and when the value is less than zero the oil forms a lenslike droplet floating on the bubble surface. It was concluded that the specific interfacial area of gas bubble available for oxygen transfer may decrease due to the oil partially covering its surface.

#### 4) Gas-oil-solid transfer:

Cells at the surface of the oil droplet may use the oxygen directly depending on the affinity of the cells for oil and on the size of the emulsified droplet. Some hydrocarbon assimilating bacteria may clump onto the surface of the oil droplet to form flocs which in turn attach to the surface of the gas bubbles. The cells take up oxygen from both organic phase and in the water film around the bubbles. It has been proposed that these cells utilise a considerable amount of oxygen from oil which spreads on the surface of air bubbles (Yoshida *et al.*, 1970).

The total oxygen transfer rate measured in a fermentation media including oil will be the sum of the oxygen transfer rates from a combination of the four ways described above.

There are many different standpoints in the literature regarding the effect of oil on oxygen transfer rates. The usual concentration of soybean oil used as a partial carbon source in antibiotic fermentations is approximately 1-20%. When this concentration is increased antibiotic production sharply decreases due to a decrease in oxygen transfer rates (Kralovcova *et al.*, 1984; Rezanka *et al.*, 1984). Stowell (1987) reported that there is a high level of residual oil at the end of a fermentation, due to physical limitation of mass transfer, and that the oil tended to increase viscosity of the culture broth and hence reduce volumetric oxygen transfer.

Rols and Goma (1989) indicated that oil, at the correct concentration, may enhance oxygen transfer rates. It was demonstrated that, at 0.28vvm and 500rpm, the use of 19% v/v soybean oil increased the value of the  $k_La$  by 1.85 fold and for smaller volumetric oil fractions  $k_La$  increases linearly with the oil loading. Due to the oxygen vector properties of soybean oil it is possible to significantly increase  $k_La$  of a bioreactor by the addition of the correct amount of lipid (Rols and Goma, 1991).

If addition of soybean oil influences the volumetric oxygen transfer coefficient then the oil must act at the gas/water interface or within the water boundary layer of the gaseous dispersion. Kawase and Moo-Young (1990) investigated the effects of oil based antifoam on mass transfer within the bioreactor system. They concluded that fermentation broth are usually non-coalescing due to surface active agents which stabilise the foam when they are formed. However, high viscosity broths tend to coalesce and therefore the additions of antifoam to such a broth would decrease the oxygen transfer rate in two ways. Firstly by interfacial blockage and therefore increased resistance to mass transfer and, secondly, by hydrodynamic change resulting in suppression of the mobility of the surface of the bubbles.

Liu *et al.* (1994) investigated the effects of lard oil, olive oil and castor oil on oxygen enhancement and as antifoam agents in a fermentation process. Addition of the oils into a baffled stirred tank decreased the volumetric oxygen transfer coefficient in the low concentration range of 0.005-0.1%. The coefficient was increased when the concentration of oil added reached 0.25%(v/v) at 400rpm.



From the literature reviewed it is seen that oxygen transfer may be increased, without changing agitation or aeration conditions within a process, by the addition of oils in the aqueous phase. However, oils require higher oxygen uptake which can then result in oxygen uptake rate becoming excessive, the concentration of dissolved oxygen will then decrease and antibiotic production reduced. The addition of oils to a fermentation offers the potential to improve efficiency of a process by increasing both antibiotic titre and improving oxygen transfer rates. Further investigation is required to fully understand the full potential of oil additions in an antibiotic producing fermentation.

## **2.6. Effect of process variables on fermentation**

In non-Newtonian, mycelial, antibiotic fermentations the detailed understanding of the influence of physical parameters on biological responses are important concepts. Studies such as media development and genetic manipulations of the organism are valuable tools but engineering aspects of a biological process are also of immense value in improving process economics and productivity.

### **2.6.1. Mixing and shear**

For mixing in a bioreactor to be efficient the impeller must sweep the entire vessel within a reasonable time and the velocity of the fluid leaving the impeller must be sufficient to carry all material to all parts of the stirred tank. Mixing is certain to be poor unless flow in the tank is turbulent. Therefore mixing can be described as a combination of three physical properties:

1. Distribution or macromixing
2. Dispersion
3. Diffusion or micromixing

Near the impeller there is a region of high turbulence where fluids converge causing intense mixing. Away from the impeller flow is slower and therefore mixing in these regions is much less intense than near the impeller. Efficient bulk mixing in a stirred tank includes circulation through the impeller mixing zones at regular intervals.

The process by which materials are transported to all regions of the vessel is called distribution or macromixing. Distribution is a slow process and is often the slowest step in the mixing process. If the impeller speed is sufficiently high then the distribution process becomes turbulent. The kinetic energy of turbulent fluid is directed into regions called eddies; masses of eddies of various sizes coexist during turbulent flow. The rapid transfer of materials throughout the vessel is facilitated by the process of breaking up bulk flow into smaller and smaller eddies. This process is called dispersion. The degree of homogeneity within a vessel is limited by the size of the smallest eddies. This is given approximately as the Kolmogorov scale of mixing, or the microscale of turbulence (Kolmogorov, 1941):

$$\lambda = (\nu^3 / \epsilon)^{0.25} \quad (2.4.)$$

where  $\lambda$  is the dimension of the smallest eddies. According to equation 2.4. the larger the vessels power input the smaller the eddies.  $\lambda$  is also dependent on the viscosity of the fluid. Within the eddies mixing relies on diffusion which over such small distances can be accomplished rapidly.

#### 2.6.1.1. Effect on morphology, growth and productivity

Mixing is one of the most important physical operations in a bioprocess, required to create optimal environmental conditions required to minimise nutrient gradients and ensure adequate flow rates at transfer surfaces. In general, the size of a typical unicellular bacteria or yeast is approximately 50-100 $\mu\text{m}$  which is usually less than the microscale of turbulence (Shamlou *et al.*, 1994). Although agitation has been reported to affect these organisms (Wase and Patel, 1985; Vrana and Seichert, 1988) it is reasonable to assume that shear does not have such a significance as when compared to the actinomycete hyphal length. There are many reports on the effects of intense agitation causing hyphal damage (van Suijdam and Metz, 1981; Mitrad and Riba, 1988; Smith *et al.*, 1990; Belmar-Beiny and Thomas, 1991). Milder agitation rates may allow elongation of the hyphae and a reduction in branching hyphae.

The influence of mixing on morphology, growth rate and yield is complicated by effects such as shear and oxygen transfer. Therefore in all experiments carried out to measure the effects of mixing the dissolved oxygen must not be allowed to fall below the critical

level for that organism. The effects of agitation are also affected by the presence of additives and toxic compounds in the medium which may affect cell wall strength and increase susceptibility to cell rupture (Shamlou *et al.*, 1994).

Studies on the effect on stirrer speed on fungal morphology have been reviewed by many different authors. Metz *et al.* (1981) studied the effects of stirrer speed on the morphology of the organism *Penicillium chrysogenum*. Morphology was characterised in terms of main hyphal length, total length, total number of hyphal tips and the hyphal growth unit. An increase in stirrer speed from 250rpm to 750rpm on a 10 litre scale caused a shift towards shorter, thicker and more branched hyphae (van Suijdam and Metz, 1981). The influence of shear stress upon hyphal length was very limited and therefore to get a substantial decrease in mean hyphal length of the organism there had to be an enormous increase in energy input. Mitard and Riba (1988) used an annular reactor to test the effect of an average shear rate on the morphology of the fungus *Aspergillus niger*. High shear rates were responsible for increased branching in a filamentous organism and that this change in morphology improved the mechanical resistance of the organism. Smith *et al.* (1990) also reported that hyphal length of *P. chrysogenum* is reduced at increasing agitation rates. Impeller tip speeds and local power dissipation rates which would have been deleterious at a 10 litre scale were acceptable at 100 litres.

Tar buck *et al.* (1985) studied the effect of variations of stirrer speed in 10 litre batch fermentations of *S. clavuligerus*. They concluded an increase in hyphal fragmentation and a decrease in clavulanic acid production when increasing stirrer speed from 375 rpm to 500 rpm. At stirrer speeds below 375 rpm there was also a decrease in productivity, which could probably be related to low oxygen transfer.

The effect of mixing and shear on morphology and clavulanic acid production in *S. clavuligerus* was also studied by Belmar-Beiny and Thomas (1991). In a 5 litre fermenter fitted with two Rushton turbines the affect of stirrer speed on cell growth and clavulanic acid production was measured. The experiments were carried out at three different stirrer speeds, 490, 990, and 1300 rpm, giving tip speeds of 1.7, 3.5, 4.6 m s<sup>-1</sup> respectively. Laboratory scale tip speeds are lower than at production scale, but the highest value, 4.6 m s<sup>-1</sup>, was close to typical production level values. Cell growth and

clavulanic acid production were found not to be very dependent on stirrer speed, it was clearly possible to obtain the same biomass concentration and clavulanic acid titre at the various agitation rates. Image analysis showed that the actual effect of increased stirrer speed in a *S. clavuligerus* fermentation was to accelerate the initial fragmentation phase of growth. The total hyphal length, main hyphal length and numbers of hyphal tips decreased at higher stirrer speeds. It was noted that *Streptomyces clavuligerus* is not very shear sensitive, which may be due to the small lengths and diameters of the organisms hyphae. No direct link between morphology and productivity was found and therefore they may be independently manipulated to increase fermenter productivity for this organism.

#### 2.6.1.2. Scale-up

Initial correlation of production rate data to parameters such as tip speed (Oldshue, 1966) and power per unit volume of bulk liquid (Einsele, 1978) at 10 and 100 litre scales gave unsatisfactory results. Therefore, the importance of the frequency at which the mycelia pass through the zones of high energy dissipation, near the impellers, and the forces to which they were exposed had to be taken into account.

Smith *et al.* (1990) proposed a model which related productivity to circulation times,  $t_c$ , and circulation frequency,  $1/t_c$ , against energy dissipation rates in the impeller region. The equation was expressed as:

$$P / d_i^3 t_c \quad (2.5.)$$

Where  $P$  is the power input and  $d_i$  is the impeller diameter. The equation suggests that the higher the frequency of mycelia passing through the zones of high energy dissipation, the more damage will occur. The model was successful and showed relationships between production rates and  $P / d_i^3 t_c$  at the different scales. Makagnisar *et al.* (1993) showed that the same parameter could be used to correlate morphological changes as well as productivity up to the 1000 litre scale. Impeller type can also be correlated into the equation (Justen *et al.*, 1996) by multiplying the power input by a given constant for impeller design.

One method of improving the scale-up strategy is to use a scale-down method. Laboratory experiments to determine operating scale parameters are carried out under

conditions that can actually be achieved both physically and chemically at the production scale. Using this approach, as long as the flow regime in both scales are the same, there is a better chance that results from the laboratory scale experiments will be reproducible on the larger scale systems.

In either scale-up or scale-down it is not possible to hold all parameters that are related to mixing constant (Oldshue, 1969). To hold the shear rates as constant as possible most fermenters have larger diameter and lower rpm. Typically large fermenter turbines have diameters that are about 0.33 to 0.4 times the tank diameter, whilst optimum turbine diameter for mass transfer has been quoted at 0.25 times the tank diameter. As the volume of the mixing vessel increases so do the lengths of the flow paths for bulk circulation. So as to keep mass transfer constant, the velocity of the fluid in the tank must be increased proportional to its size.

Mass transfer can hardly ever be applied to scale-up in this manner as mass transfer will inevitably decrease with scale. If power input to vessel volume ratio is kept constant during the scale-up mass transfer may be increased in proportion to the vessel diameter. This may be explained using equation 2.6.

$$P / V_L \propto \cancel{V_L} \omega_i^2 \quad (2.6.)$$

### 2.6.2. Dissolved oxygen tension

A most critical operation in an aerobic fermentation is the dissolution of oxygen required for the respiration of microbes. Molecular oxygen is required by aerobic microorganisms as an oxidant to meet their requirements of energy. The <sup>RATE AT WHICH</sup> ~~velocity of~~ <sup>OXYGEN IS CONSUMED</sup> respiration, and consequently the rate of microbial growth, will depend on the concentration of reactants in the medium. In contrast to most other reactants oxygen has a very low solubility which is further decreased by dissolved electrolytes and organic substrates within the culture media. It is considered that respiration rates become independent of the concentration of dissolved oxygen above a certain critical value which will depend on the specific growth rate of the organism. Maximum biomass production is achieved by maintaining the concentration of dissolved oxygen above this critical value. The rate of oxygen transfer to the liquid is highly dependent upon agitation speed, the size and design of the fermenter, the quality of medium added to the fermenter and the viscosity of the broth. The objective of the majority of fermentations

is the product rather than the organism itself and therefore aeration conditions necessary for optimum production rates will not necessarily be the same as that required to produce biomass.

In a fermenter the air is usually sparged into the media below the bottom of the impeller so that when the air stream meets the high shear field created by the impeller and the stream is broken up into finely dispersed bubbles. Typically a sparger ring is used that is 0.8 times the turbine diameter. Due to the high power input from the impeller the design of the sparger is not critical to aeration. The range of air flow rates used within a conventional fermenter is 0.5 - 1.5 vvm. Increasing air flow rates may result in flooding, where the impeller is rotating in a gas phase and therefore cannot transfer gas to solution. Agitation delays escape of air bubbles from the liquid, prevents air bubbles coalescing and decreasing the thickness of the liquid film at the gas-liquid interface by creating turbulence in the medium. The finely dispersed bubbles created by the impeller create a large surface area for the oxygen to diffuse into the liquid phase. Oxygen transfer in a fermentation is generally described as:

$$\text{OTR} = k_L a (C^* - C_L) \quad (2.7.)$$

$K_L$  is the liquid phase mass transfer coefficient and  $a$  represents the gas-liquid interfacial area per unit volume dispersion.

#### 2.6.2.1. Effects on morphology, growth and productivity

The effect of dissolved oxygen on productivity and morphology of filamentous fermentations is well studied. Zetelaki and Vas (1968) found that, when using oxygen instead of air in *Aspergillus niger* fermentations, there was a considerable decrease in suspension viscosity even though the biomass concentration was higher. They also found that the cell walls in oxygenated cultures were thinner and more flexible, which could explain the reduced viscosity. The effect of oxygen concentration in 10 litre *P. chrysogenum* fermentations has been studied by van Suijdam and Metz (1981). Aeration with oxygen instead of air produced shorter, thicker, highly branched and more tapered hyphae.

Vardar and Lilly (1982) modified a 7 litre fermenter to simulate the dissolved gas and hydrostatic pressure gradients of larger vessels to investigate the effects of cycling

dissolved oxygen on penicillin production. Below 30% air saturation the specific penicillin production rate decreased sharply and no production was observed below 10%. Oxygen uptake of *P. chrysogenum* was significantly affected below 7% DOT which demonstrates that the critical DOT values for penicillin production and oxygen uptake are two independent parameters. Cycling the DOT around the critical level for penicillin production caused a considerable decrease in specific production rates. This decrease was reversible indicating a shift in cell metabolism.

In general, Streptomycete fermentations are reported to be more tolerant to low dissolved oxygen levels than penicillin fermentations, but prolonged low levels will ultimately be harmful (Bader, 1986).

Scott *et al.* (1988) studied the effects of oxygen concentration on clavulanic acid biosynthesis from immobilised *Streptomyces clavuligerus*. They claimed that at the different levels of oxygen used during the experiment the effect on the rate of clavulanic acid produced was due to the oxygen levels affecting the enzymes involved in the clavulanic acid biosynthetic pathway. Yegneswaran *et al.* (1988) also studied the effects of reduced oxygen on the growth and antibiotic production of *S. clavuligerus* in batch cultures using a defined media. Antibiotic titre was unaffected by reduced oxygen levels for the first 50 hours of the fermentation. After growth had ceased, antibiotic concentration decreased by a factor of approximately three under reduced oxygen, while antibiotic concentration was stable when air was used. The conclusion from this study was, again, that the enzymes responsible for hydrolysing antibiotics may be regulated by aeration conditions.

Rollins *et al.* (1988) found that if the level of dissolved oxygen was decreased to almost zero for approximately 10 hours during the rapid growth phase of *S. clavuligerus* in 10 litre fermentations, production of cephamycin C was delayed. Controlling the dissolved oxygen at 50% or 100% throughout the fermentation process elevated antibiotic levels by 2-3 fold but did not significantly alter the specific growth rate of the culture. These results demonstrated that controlling dissolved oxygen levels during periods of rapid growth markedly improves efficiency of cephamycin C production in *S. clavuligerus*.

In large scale fermentation under dissolved oxygen control, localised depletion in the oxygen levels and mass transfer difficulties are expected. Therefore, although the 10 litre scale demonstrates that antibiotic synthesis in *S. clavuligerus* can recover from relatively long periods of oxygen starvation, in large fermentations the cells are likely to experience short repeated interruptions in oxygen supply. Production scale fermentations simulated by an airlift system have shown that Streptomyces cells are sensitive to rapid changes in dissolved oxygen (Pollard, 1996). Cell metabolism is affected by the heterogeneity of the dissolved oxygen to a greater extent than the average oxygen tension experienced in one circulation of a vessel.

Yegneswaran *et al.* (1991a) studied the effects of oxygen fluctuations in 2 litre fermentations. An experimental Monte Carlo method was applied; air being supplied to the culture in random cycles following a lognormal distribution to model circulation within industrial production scale vessels. Comparable experiments were also carried out with constant period cycling of air and with a continuous air supply. The yields of cephamycin C and penicillin N were suppressed by the Monte Carlo method when compared to constant period cycling. It was concluded that the biosynthetic enzymes, which are sensitive to oxygen levels were likely to be affected not only by the mean time of cycling but also distribution of cycles.

Yegneswaran *et al.* (1991b) used a proportional integral control system to control dissolved oxygen during fermentation at constant shear and mass transfer conditions. The growth and antibiotic production of *S. clavuligerus* was studied at different dissolved oxygen levels during the fermentation using three different methods. The first of these methods was no oxygen control which would be used as a control to the experiment. The second method was that oxygen would be controlled at a preset saturation level throughout the fermentation. The final protocol was that oxygen was controlled at a high level only during the growth phase of the fermentation. When compared to the control an approximate 2 fold increase in antibiotic level was seen when the dissolved oxygen was controlled at a saturation level during growth phase. A decrease in specific growth rate and cephamycin C yields was observed when the dissolved oxygen was controlled at 50% during the fermentation. It was concluded that the most successful control strategy was to control dissolved oxygen only during active



growth when the biosynthesis enzymes involved in production of the antibiotic are synthesised.

Unfortunately, the yield of antibiotic does not generally provide a reliable indication of the activity of the biosynthetic enzyme systems as additional mechanisms may be involved in the regulation of production. A study by Rollins *et al.* (1990) concluded that when the dissolved oxygen was maintained at saturation level during batch fermentation of *S. clavuligerus* accumulation of intermediate penicillin N was lowered whilst formation of the end product, cephamycin C, was increased relative to a fermentation without dissolved oxygen control. It appeared that oxygen derepression of deacetoxycephalosporin C synthase and isopenicillin-N-synthase are important regulatory mechanisms.

#### 2.6.2.2. Scale-up

As reported earlier the rate controlling step in oxygen uptake rate is often oxygen transport from the gas-liquid phase interface to the bulk of the liquid (Hubbard *et al.*, 1994). The effect of mass transport must be included when scaling-up a process. This may be done by maintaining the oxygen uptake rate per unit vessel volume, (OUR/V), in each of the scale-up systems:

$$\text{OUR/V} = k_L a (C^* - C_L) \quad (2.8.)$$

A correlation between mass transfer coefficient and operating variables, fluid properties and geometrical variables is needed so as to implement the above scale-up strategy. Typically transfer coefficients are expressed as a function of power input and superficial gas velocity, Equation 2.2.  $\alpha$  and  $\beta$  are variable dependent on reactor size (Mooyman, 1987). Equation 2.2. is of limited value and a formula independent of fermenter size is required. Selecting a particular  $k_L a$  correlation with scale will mean assuming that the correlation will apply to whatever process conditions are developed. As most fermentation broths contain macromolecules, such as proteins, a mass transfer coefficient correlation selected as part of the scale-up procedure should apply to Non-Newtonian and viscoelastic broths. Mass transport must be governed by the same mechanisms at all different scales of fermentation.

### 2.6.3. Carbon dioxide concentration

Carbon dioxide is the major product of cell carbohydrate metabolism which is released into fermentation broths. Carbon dioxide plays an important role in primary metabolism and appears to have a strong effect on secondary metabolism (Bader, 1986). In production scale fermentation carbon dioxide evolution can easily lead to an increase in local gas concentration as mixing times may usually be in the order of several minutes. This may lead to significant differences in total pressure from the top to the bottom of the process vessel. The level of dissolved carbon dioxide will therefore vary throughout a production scale vessel and expose the organisms to higher and much more varied partial pressures of carbon dioxide. These varying levels may be inhibitory to the cells metabolism which, in turn, may affect morphology and productivity of the organism.

Carbon dioxide and hydrocarbonate ions have both been shown to affect the membrane of the cell. Jones and Greenfield (1982) demonstrated the effect of carbon dioxide and hydrocarbonate ions on membrane structure. They concluded that carbon dioxide and hydrocarbonate have different sites of action on the membrane of the cell. Dissolved carbon dioxide primarily affecting the fatty acid core of the membrane and the hydrocarbonate affecting the charged phospholipid head groups and proteins at the surface of the membrane. These affects will therefore change the optimum fluidity and surface charge density radically changing the properties of the membrane. These changes have been implicated in <sup>VARIAIONS</sup>changes in cell growth.

An increase in partial pressure of carbon dioxide has been reported to result in anaesthesia (Pirt and Callow, 1958) a process occurring when the membrane volume contains a critical concentration of carbon dioxide within the lipid phase. This carbon dioxide changes the fluidity of the membrane (Pang *et al.*, 1980) consequently changing membrane transport which in turn will affect morphology of the organism.

#### 2.6.3.1. Effect on growth and productivity

Pirt and Callow (1958) showed that increased partial pressures of carbon dioxide in a fermentation of *Aerobacter aerogenes* reduced sugar utilisation with little effect on cell yield. Ishizaki *et al.* (1971) reported that increased levels of carbon dioxide stimulated both growth and yields from a culture of *Bacillus subtilis*, but substantially reduced production of iosine. In a later paper, Ishizaki *et al.* (1973a) conclusively reported that it

was the partial pressure of carbon dioxide affecting the fermentation and not the level of bicarbonate in the medium. Ishizaki *et al.* (1973b) also determined that below the critical level of 0.05 atm the fermentation was not affected. These studies indicate that increased partial pressures of carbon dioxide stimulate growth at the expense of product formation. This was highlighted by Nash (1974) for an antibiotic producing streptomycete fermentation. Increasing partial pressures of carbon dioxide had little effect on growth but reduced production of erythromycin by *Streptomyces erythraeus* substantially.

The effect of dissolved carbon dioxide on morphology of *P. chrysogenum* in penicillin fermentations was examined by Smith and Ho (1985). Light microscopic studies indicated that the morphology of *P. chrysogenum* was subject to change when exposed to various levels of inlet carbon dioxide in the medium. At low influent partial pressures of carbon dioxide in the vessel, 3-5%, the morphology was predominantly filamentous. If the influent partial pressure of carbon dioxide was increased to 13-20%, swollen, stunted hyphae predominate with a proportion of spore like cells present. Scanning electron microscopy studies revealed, and confirmed, that the morphology was subject to change while the fermentation was sparged with gases of various carbon dioxide concentrations.

The effects of carbon dioxide on the rheological behaviour and oxygen transfer of *P. chrysogenum* in submerged fermentations was carried out by Ju *et al.* (1991). Rheological behaviour of the culture broth was found to change significantly with exposure to high levels of carbon dioxide. This was attributed to a wide variation in morphology, as seen by Smith and Ho (1985). Rheological behaviour of the culture exposed to 5% carbon dioxide significantly changed the flow behaviour index,  $n$ , fluid consistency index,  $K$ , and apparent viscosity, when compared to the control fermentation. This was assumed to be as a direct consequence of the filamentous nature of *P. chrysogenum*. Similar patterns with regards to rheological parameters over the time course of a fermentation have been observed in Streptomycete fermentations (Tuffile and Pinho, 1970). From experiments carried out by Ju *et al.* (1991) results show that at 20% carbon dioxide the flow behaviour index was always found to be greater than 0.7 due to pellet formation.

Ju *et al.* (1991) concluded that while the oxygen diffusion coefficient and solubility did not change significantly, rheological properties and oxygen transfer coefficient were found to change significantly during the course of fermentation at different partial pressures of carbon dioxide. They developed a correlation relating volumetric oxygen transfer coefficient with effective oxygen diffusion coefficient and apparent viscosity.

$$k_L a = A De^{2/3} \mu_{app}^m \quad (2.9.)$$

A and m are determined by plotting  $k_L a / De^{2/3}$  against  $\mu_{app}$  logarithmically.

Bylinkina *et al.* (1973) observed the effects of carbon dioxide levels of upto 40% of saturation on *S. aureofaciens* and *S. antibioticus*. They concluded that above 15-20% of saturation the level of carbon dioxide inhibited respiration to approximately half the value at 0% inlet carbon dioxide. Normal respiration rates were established when carbon dioxide inlet was reduced. The authors also reported that increasing the levels of dissolved carbon dioxide inhibited streptomycin production from *S. griseus*.

The effect of carbon dioxide tension on the morphology and clavulanic acid production of *S. clavuligerus* in 5 litre batch stirred tank fermentation has been studied by Belmar-Beiny and Thomas (1992). Results concluded that increasing influent carbon dioxide reduced clavulanic acid titres. At 8% and 18% v/v influent carbon dioxide a reduction in mean number of hyphal tips was observed when compared to the control of 0.04%. Also at 18% v/v carbon dioxide it was seen that there was less branching at the earlier stages of growth when compared to the control. Observations from these experiments differ from those described for *P. chrysogenum* where more branches were formed at the increased carbon dioxide levels. Swollen fungal hyphae were also observed for *S. clavuligerus* at the higher carbon dioxide levels. It was concluded that the decrease in clavulanic acid titres may therefore be due to changes in morphology of the organism at the 8% v/v level. Production may be related more to growth inhibition at the 18% v/v carbon dioxide level than morphological changes as production of clavulanic acid did not start until fragmentation had ended.

### 2.6.3.2. Scale-up

The effects of carbon dioxide on the scale-up of fermentations is not very well understood. Partial pressures of carbon dioxide in a shake flask experiment will differ substantially to those in a laboratory fermenter that is continuously sparged with air. Early growth will proceed faster and antibiotic production repressed in a shake flask if increasing carbon dioxide levels stimulate cell growth and inhibit productivity. Partial pressures of carbon dioxide in pilot plant scale will also be substantially different to those at the production scale. Pilot plant fermenters have a limited liquid depth whereas production vessels may be meters deep. Depth creates hydrostatic pressure at the bottom of a production vessels which will increase the solubility of carbon dioxide. Also, on production scale, the volume of air per unit volume of liquid is reduced, further increasing carbon dioxide levels. The exposure of the organisms to such high levels of carbon dioxide in a production scale vessel will be periodic rather than continual. Therefore, an increase in over-pressure on a large vessel, for example to improve oxygen transfer rates, might result in deleterious effects to the fermentation due to high carbon dioxide concentrations.

Some of the differences that occur between scales can be offset by adjustments to the fermentation media such as addition of calcium carbonate to supply a slow release of carbon dioxide during the initial stages of the growth phase (Bader, 1986). Production scale fermentations may also be started with a low air flow rate and low agitation speed in order to decrease power costs and increase carbon dioxide levels. These parameters will be increased in later stages of the fermentation to supply oxygen and increase carbon dioxide removal rates. Additional back pressure can also be used to alter carbon dioxide levels in the larger scale fermentations.

## **2.7. Summary**

This literature survey highlighted the industrial significance of actinomycetes, *S. clavuligerus* and clavulanic acid. The literature showed that oils are supplemented to a fermentation medium in a variety of processes in order to increase secondary metabolite titres. The use of lipid supplements has many advantages over carbohydrate only carbon sources. There are also disadvantages to the use of oils, including the oil remaining at the end of batch fermentation. It is clear that there are many parameters within a fermentation that may affect the utilisation of lipids within a complex medium. The review showed that although many studies have found residual lipid to be of concern little research is being carried out in order to investigate reasons behind, and ways to reduce, residual oil levels. This study will investigate both physical and chemical parameters within a 5 litre scale fermentation of *S. clavuligerus* in order to reduce residual oil levels with the aim of increasing clavulanic acid titres.

### **3. MATERIALS AND METHODS**

#### **3.1. *Streptomyces clavuligerus* fermentations**

##### **3.1.1. Organism**

An isolate of *Streptomyces clavuligerus* ATCC was supplied by SmithKline Beecham Pharmaceuticals (Worthing, West Sussex, U.K.).

##### **3.1.2. Media suppliers**

The suppliers of the major media components are listed in Table 3.1. Chemicals were also generously obtained from SmithKline Beecham Pharmaceuticals (Worthing, West Sussex, U.K.).

##### **3.1.3. Fermentation equipment**

###### **3.1.3.1. Fermenter vessels**

Initial media variation fermentations were carried out using a glass 7 L capacity LH series 210 fermenter, (Inceltech/LH Fermentation Ltd, Reading, Berks., U.K.), with a 5 L working volume. The top plate contained eight openings for electrodes, acid/base and antifoam addition and air inlet and outlet. The base plate contained a steam sterilizable sample port, electric heater and temperature probe. Three equally spaced six bladed Rushton turbines were mounted in the stirrer shaft and the vessel contained four equispaced tank baffles, (Figure 3.1.). Dissolved oxygen and pH electrodes were steam sterilizable insertion probes supplied by Ingold, (Ingold, Life Sciences Laboratories, U.K.). Further experiments were carried out using a glass 7 L capacity LH series 2000 fermenter, (Inceltech/LH Fermentation Ltd, Reading, Berks., U.K.), with a 5 L working volume. The top plate and base plate were as for the 210 series except the sample port was connected to the top plate. DOT was maintained above a limiting level by increasing stirrer speed or, where reported, by blending oxygen in to the inlet air.

**Table 3.1. Suppliers and grades of media components and chemicals**

<b>Media Component</b>	<b>Supplier</b>	<b>Grade</b>
Acetyl chloride	BDH	AnalaR
Ammonia	BDH	AnalaR
Ammonium acetate	BDH	HPLC grade
Ammonium sulphate	BDH	AnalaR
Antifoam	SmithKline Beecham	-
Benzene	BDH	AnalaR
Calcium carbonate	BDH	AnalaR
Calcium chloride	BDH	AnalaR
Clavulanic acid salt	SmithKline Beecham	-
Dextrin	Sigma	T III - Corn
Ethanol (absolute)	BDH	AnalaR
Glacial acetic acid	BDH	HPLC grade
Glycerol	BDH	AnalaR
Gum arabic	BDH	AnalaR
Hydrochloric acid	BDH	AnalaR
Magnesium sulphate	Sigma	AnalaR
Methanol	BDH	HPLC grade
Orthophosphoric acid	BDH	AnalaR
Phenol	BDH	AnalaR
Potassium carbonate	BDH	AnalaR
Potassium dihydrogen orthophosphate	BDH	AnalaR
Rapeseed oil	SmithKline Beecham	-
Sodium chloride	BDH	AnalaR
Sodium hydroxide	BDH	AnalaR
Sodium hypochlorite	BDH	Spectros.
Sodium nitroprusside	BDH	AnalaR
Soya flour	SmithKline Beecham	-
Sulphuric acid (conc.)	BDH	AnalaR
Thiosulphate Ringers	Oxoid	-
Technical agar no. 3	Oxoid	-
Tributylin	Aldrich	-
Vanillin	Aldrich	-
Zinc sulphate	Sigma	AnalaR

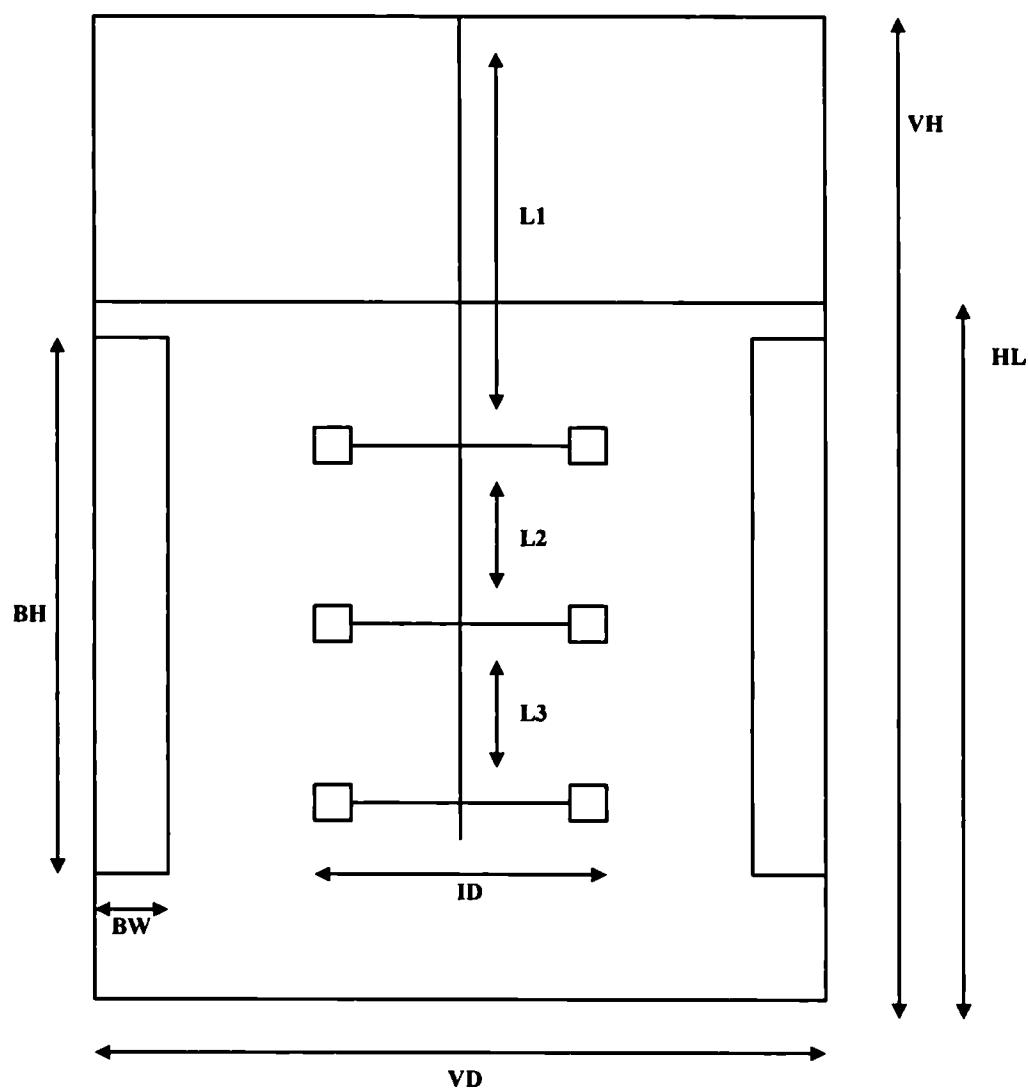
Aldrich Chemical Company, New Rd, Gillingham, Dorset, SP8 4JL, U.K.

BDH, Merck Ltd, Merck House, Poole, Dorset, BH15 1TD, U.K.

Oxoid, Unipath Ltd, Wade Rd, Basingstoke, Hampshire, RG24 0PW, U.K.

Sigma Chemical Company, Fancy Rd, Poole, Dorset, BH 17 7BR, U.K.





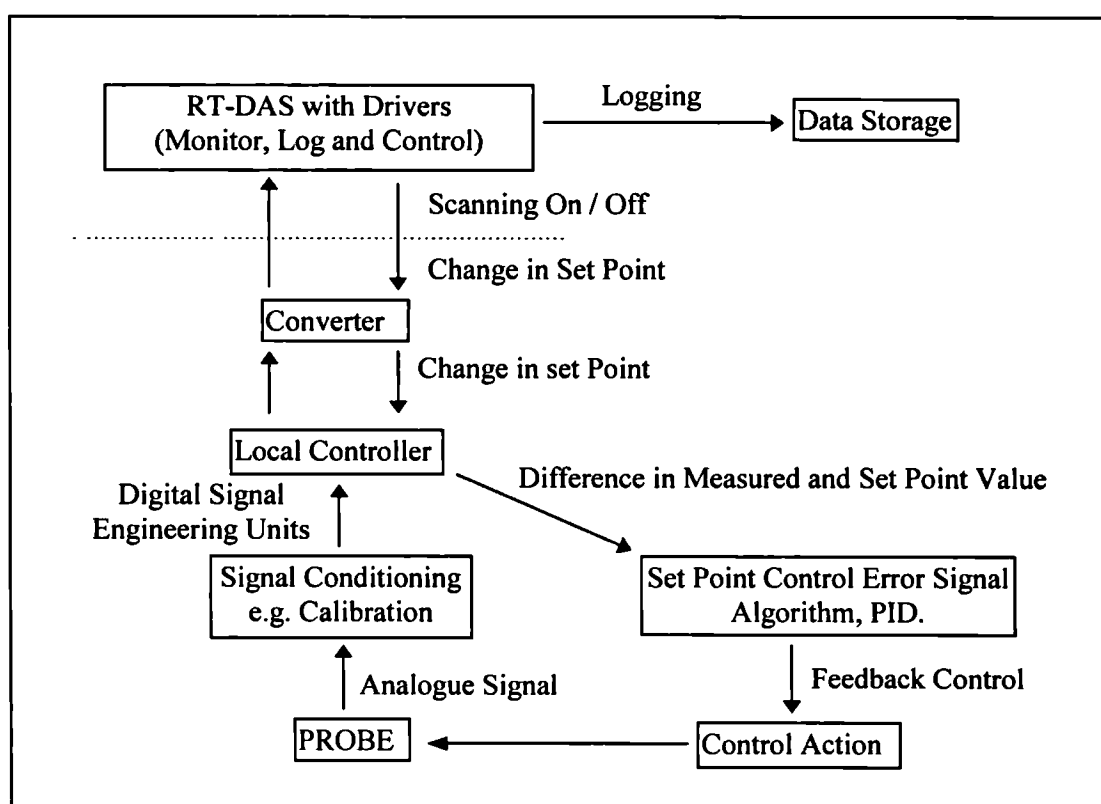
Symbol	Definition	LH210 (m)	LH2000 (m)
VH	Vessel Height	0.41	0.41
VD	Vessel Diameter (inside)	0.16	0.16
HL	Liquid Height	0.28	0.28
L1	Shaft length to first impeller	0.2	0.16
L2	Length between first and second impeller	0.09	0.075
L3	Length between second and third impeller	0.08	0.075
ID	Impeller Diameter	0.07	0.06
BH	Baffle Height	0.305	0.305
BW	Baffle Width	0.02	0.02

Figure 3.1. Fermenter dimensions

### 3.1.3.2. Off gas analysis and data logging

Off gas analysis was carried out through a VG Prima mass spectrometer (VG Gas Analysis Ltd, Cheshire, U.K.). This was used to measure carbon dioxide, oxygen, nitrogen and argon in the fermenter off gas every three minutes. The gas analysis was connected through the RT-DAS system (Acquisition systems Ltd., Sandhurst, Surrey, U.K.) and the data processed so as to record oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER), of units  $\text{mmol L}^{-1} \text{h}^{-1}$ , and the respiratory quotient (RQ).

Data logging was carried out using the RT-DAS program. RT-DAS control architecture is shown in Figure 3.2. Analogue signals from fermentation probes are conditioned by specialised control modules. The set point control is also an integral part of the control. Feedback control takes place depending on the signal of error, the difference between a measured value and the set point, and the appropriate action is then taken.



**Figure 3.2. RT-DAS control model**

### 3.1.4. Spore preparation medium

All media component quantities are given as a ratio relative to the level of modified starch, i.e. 3:1 represents three parts oil to one part starch. Composition of the spore preparation medium, Table 3.2., was supplied by SmithKline Beecham Pharmaceuticals. Media was adjusted to pH 7.0 and sterilised at 121°C for 20 mins in a Denley bench autoclave (Patterson Scientific Ltd, Dunstable, Bedfordshire, U.K.). Before cooling media was poured into sterile petri dishes and stored at 4 °C.

**Table 3.2. Composition of spore preparation medium**

Component	Ratio
Modified starch	1
Potassium dihydrogen orthophosphate	0.1:1
Magnesium Sulphate. 7 hydrate	0.1:1
Sodium Chloride	0.1:1
Ammonium Sulphate	0.1:1
Calcium Carbonate	0.4:1
Trace Elements	1:1
Agar, (no. 3)	1.5:1

### 3.1.5. Spore production and storage

An isolate of *Streptomyces clavuligerus* was grown on solid agar plates, of the composition shown in Table 3.2., at 28 °C for 10 days. The spores were harvested in 20% glycerol and stored at -70 °C.

The concentration of spores was counted using a Bacteria Counting Chamber. 10 µL of a 10x dilution of spore preparation was placed on the grid of the counting chamber and a cover slip placed on top. The number of spores were counted within the 25 small square grid. This number was multiplied by a 10<sup>4</sup> factor (as stated in the manual) and multiplied by the initial dilution.

### 3.1.6. Spore stock sterility control

A spore stock sterility test was carried out at six monthly intervals. An inoculation loop of spore suspensions was streaked on solid media, as in Table 3.2. The plates were then incubated at 28°C for 3-5 days. An inoculation loop of spore stock was also inoculated on to nutrient agar (no. 3) and incubated at 37°C for 3-5 days. The spore suspension would be classed as sterile if no contamination was seen in either test.

### 3.1.7. Seed preparation

Seed medium was prepared as in Table 3.3., adjusted to pH 7.0 and sterilised in a Denley bench autoclave at 121°C for 20 mins.

**Table 3.3. Composition of seed medium**

Component	Ratio
Soya flour	2:1
Modified starch	1
Rape seed oil	0.5:1
Potassium dihydrogen orthophosphate	0.06:1

### 3.1.8. Inoculum preparation

500 µL of a  $2 \times 10^6$  spores mL<sup>-1</sup> *Streptomyces clavuligerus* glycerol stock spore preparation was used to inoculate duplicate 2 L baffled flask containing 200 mL of seed media, Table 3.3. The flasks were then incubated for 60 h at 28 °C on an ISF-1-V orbital shaker (Adolf Kühner, Schmeiz, Switzerland) at 200 rpm with a throw diameter of 0.10 m. Inocula transfer was considered a critical process parameter and was monitored closely. Before inoculation the viscosity and pH of the seed were noted.

### 3.1.9. Process medium

4.8 L of process medium, Table 3.4., was steam sterilised *in situ* at 121 °C, 1 Bar for 20 mins. Media was adjusted to pH 7.0 before inoculation with 30% ammonia solution.

**Table 3.4. Composition of process medium**

Component	Ratio
Soya flour	3:1
Modified starch	1
Rape seed oil	2.3:1
Phosphate	0.12:1
Antifoam	0.1:1
Trace elements	1:1

**3.1.10. Process conditions**

Fermentation conditions are described in Table 3.5. Dissolved oxygen tension was maintained above 50% for a number of fermentations by mixing oxygen into the air inlet sparger. All fermentations were run for 160 h.

**Table 3.5. Process parameter set points**

Parameter	Set point
Temperature	26°C
pH	7.0
Dissolved Oxygen Tension	>50%
Pressure	-
Air flow rate	1 vvm
Stirrer speed	625 rpm

## **3.2. Biomass Measurements**

### **3.2.1. Viscosity**

Due to the turbidity and particulate nature of the soya flour and oil complex media traditional biomass analysis through optical density and dry weight measurements were not possible. Protein measurements were not carried out for biomass quantitation due to the presence of protein in the soya flour. The increase in viscosity of media throughout the fermentation was used as a measure of biomass. Viscosity was measured using a Wells-Brookfield cone/plate digital viscometer (Brookfield Viscometers Ltd., Loughton, Essex, U.K.).

#### **3.2.1.1. Principle of operation**

The Wells-Brookfield cone/plate digital viscometer rotates a conical spindle at a precise speed and measures the torque necessary to overcome the viscous resistance to movement caused by the sample located between the spindle and a flat stationary plate. The spindle is driven by a calibrated beryllium copper spring and the degree to which the spring is wound is proportional to the viscosity of the fluid. The viscometer is calibrated so that the cone and plate are a set distance apart.

#### **3.2.1.2. Initial set-up**

The viscometer was mounted securely onto a laboratory stand and levelled referring to the bubble level on the back of the instrument. The viscometer sample cup was connected to a water bath to maintain the sample at 26°C.

#### **3.2.1.3. Calibration**

The viscometer and water bath were turned on and allowed to equilibrate to 26°C. The cone spindle was screwed on avoiding side thrust on the shaft. The mating surfaces of the spindle and the lower shaft were cleaned thoroughly to prevent off-centre rotation of the spindle. The power was switched on and speed set at 10 rpm. The reading was allowed to stabilise and then the reading was adjusted to zero using the zero dial. The motor was switched off and the sample cup placed against the adjusting ring. The viscometer was run at 10 rpm and the adjusting ring was turned to the left until the reading stabilised at or near zero. The adjusting ring was turned in small increments to the right until the reading fluctuated. This determined the point at which the pins made

contact. The adjusting ring was then turned to the left exactly the width of one minor division mark on the adjusting ring. The pins are separated by exactly 0.0005 inches. Calibration was carried out before every sample.

#### 3.2.1.4. Operation

The viscometer was zeroed and calibrated as described above. The viscometer was switched off and 1 mL of sample was placed in the sample cup. The sample volume must be sufficient to wet the face of the spindle and approximately 1.0 mm up the spindles outside edge. The sample cup was replaced and the viscometer switched on. The sample was allowed to equilibrate to 26°C. Readings were made at all rotational speeds.

The viscosity was calculated, as stated in the operation manual, as follows:

$$\text{Viscosity} = \text{Display reading} \times \text{Factor}$$

$$\text{Factor} = \text{Range} / 100$$

**Table 3.6. Range values to calculate viscosity.**

Speed (rpm)	Shear rate (sec <sup>-1</sup> )	Range
100	384.0	64
50	192.0	128
20	76.8	320
10	38.4	640
5.0	19.2	1280
2.5	9.6	2560
1.0	3.84	6400
0.5	1.92	12800

### 3.2.2. Biomass Monitor

The Biomass Monitor (Aber Instruments, Aberystwyth, UK.) is an on-line method of measuring biomass based on the dielectric properties of suspended cells, such as increase in capacitance. Capacitance, measured in picoFarads (pF) increases with cell biomass during the fermentation due to the increase in the number of cell walls that are available for polarisation. Sarra *et al.* (1996) has already described the relationship between biomass concentration using the capacitance based biomass probe with rheology and morphology on *Saccharopolyspora erythraea* fermentations.

#### 3.2.2.1. Equipment

The instrument used in all fermentations was the Biomass Monitor, model 214, dual frequency version. This monitor was externally controlled by a Z-star 433 L microcomputer (Zenith Data Systems) which also records all data. All measurements were carried out with the standard 270 mm x 25 mm probe inserted into a top plate port. The probe was sterilised *in situ* at 121°C for 20 minutes. The signal from the probe was amplified using a head amplifier and then transferred to the biomass monitor. The instrument was set on low range and the low pass filter set at 300 s to enable biomass monitoring in low conductance media, (2-16 mS). The frequency was set at 0.45 M Hz.

#### 3.2.2.2. Check crosstalk

Cross talk is where there are changes in the measured capacitance values due to changes in the conductance of the media. It is important to check that crosstalk is kept to a minimum level. The probe was connected to the head amplifier and placed in a solution of potassium chloride with an approximate conductance of 16 mS. Frequency mode was set on single, the range on low and the low pass filter off. The probe was then cleaned with the clean cycle 4-5 times. Frequency was set at 0.2 M Hz and the capacitance read. Frequency was changed to 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 M Hz and the capacitance read at each frequency. Conductance of the solution was then changed to 10 mS and 4 mS by dilution of the original 16 mS solution with water, and the method above repeated. Results were plotted and any cross talk noted. If crosstalk was noted solutions are found in the operators manual.



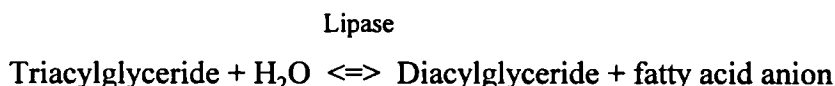
### 3.2.2.3. Initial set-up

Before sterilisation the instrument was switched on and allowed to warm up for a minimum of 30 minutes. The front panel error light should change from yellow to off. The earth wire must also be connected to the fermenter. Prior to inoculation, after sterilisation, the fermenter was set at the run temperature with no aeration or agitation. Frequency was set at 0.45 M Hz and the display set to  $\Delta C$ . The capacitance display was adjusted to zero using the 'offset' control. The instrument was switched to dual frequency mode and the 'set medium' adjusted to zero, with the LED between the red and green stages. With the aeration and agitation at their set points for the fermentation the 'gain balance' was adjusted so the  $\Delta C$  display again reads zero. The monitor was then logged on to the microcomputer and data collected throughout the fermentation.

## 3.3. Analytical methods

### **3.3.1. Lipase assay**

The principle of this assay is based on lipase hydrolysis of emulsified triacylglycerides according to the reaction:



There are many methods used to detect lipase activity (Jensen, 1983) which have been compared (Vorderwulbecke *et al.*, 1992). By titration at the optimum pH the formation of free fatty acids per unit of time can be measured and the amount of titrant consumed to maintain a constant pH will be a measure of lipase activity. For *Streptomyces clavuligerus* the reaction is maintained at a pH of 7.2 and room temperature (Cavanagh, 1996). Lipase, EC 3.1.1.3., hydrolyses emulsified triacylglycerides at the interface between the oil droplet and the aqueous phase. Therefore the degree of emulsification will also play an important part in establishing activity.

#### 3.3.1.1. Reagents

Reagents were made as in Table 3.7. The titrant of 0.01M NaOH was stored at room temperature and used within one month. Emulsification reagent was made as in Table 3.7. and stored at 4 °C for up to one month. Fresh substrate reagent was prepared daily.

The components were placed in a bench top blender and mixed at high speed for 20 seconds. The reagent was kept stirring on a magnetic stirrer (supplied by Aldrich) on setting 4 at room temperature to avoid separation of the substrate.

### 3.3.1.2. Assay

20mL of substrate reagent was placed in a thermostatically controlled Titrilab pH stat vessel comprising of a VIT 90 video titrator, ABU 93 titraturette and SAM 90 sample station, (Radiometer-Copenhagen, Denmark.) The reagent was allowed to equilibrate to room temperature. 1mL of sample was added and the reaction vessel was mounted in the titration assembly. The pH of the emulsion was adjusted to 7.2 using 0.01M sodium hydroxide. Automatic titration with 0.01 M sodium hydroxide was allowed to continue for 10mins. Lipase activity was calculated from the rate of addition of alkali and expressed as lipase units per mL of sample. The consumption of 1 mL of 0.01 M NaOH is equivalent to the liberation of 10  $\mu$ mole of fatty acid. It follows that lipase activity is calculated as:

$$\text{Lipase Activity (Units mL}^{-1}\text{)} = (\text{volume of NaOH consumed} \times 10) / \text{time}$$

1 Unit  $\text{mL}^{-1}$  was defined as the release of 1 mmole of titratable fatty acid per minute under the assay conditions used.

**Table 3.7. Composition of reagents for lipase assay**

Reagent	Amount	Component
Titratant	-	0.01 M Sodium hydroxide
Emulsification Reagent	17.9 g	Sodium chloride
	0.41 g	Potassium dihydrogen orthophosphate
	400 mL	De-ionised water
	540 mL	Glycerol
	6.0 g	Gum arabic
Substrate Reagent	15 ml	Tributyryn
	235 ml	Deionised water
	50 mL	Emulsification reagent

### **3.3.2. Total lipid analysis**

The aim of the total lipid assay is to measure the amount of total lipid remaining in the fermentation broth. This assay was based on the Boehringer test combination, (Boehringer Mannheim Ltd., East Sussex, U.K.). The principle of the assay is that all lipids react with sulphuric and phosphoric acids and vanillin to form a pink-coloured complex. The extent of pink colour can be measured spectrophotometrically at 536 nm.

#### **3.3.2.1. Vanillin reagent**

1.98 g vanillin was dissolved in 40 mL of absolute ethanol made up to 100 mL in a volumetric flask with de-ionised water. This was then made up to 1 L in a volumetric flask with concentrated orthophosphoric acid and stored in a 1 L light proof container for approximately one month. This reagent was initially pale yellow in colour and was not used if it appeared a brown/dark pink colour.

#### **3.3.2.2. Preparation of standards**

Standards of known rapeseed oil concentration were prepared in absolute ethanol so as to calculate the final concentration in a sample. Each standard was weighed out in plastic weighing boats on a Sartorius four decimal place balance. The rapeseed oil was transferred to volumetric flasks using a pasteur pipette. The weighing boat was then rinsed with absolute ethanol and poured into the flask. The volumetric flasks were then filled with absolute ethanol. Standards were assayed in triplicate with each batch of samples assayed.

#### **3.3.2.3. Assay**

Whole broth samples and standards were warmed to 30°C in a water bath for 30 mins prior to the assay. Samples were then mixed in turn on a bench Whirlmixer. 50 µL of each sample was added to 2 mL of concentrated sulphuric acid into clear 10 mL glass test tubes. A blank was prepared at the same time, this was as for the sample assay minus the broth. All samples were assayed in triplicate. Tubes were then covered with a plastic marble and boiled at 100°C for 10 minutes in a water bath. After 10 minutes cooling in tap water, the tubes were mixed on a bench top Whirlmixer. 100 µL of each triplicate reaction mixture was added to 2.5 mL of vanillin reagent, and left to incubate at room temperature for 30 mins, mixing each tube every 10 minutes during this time. The sample was assayed for lipid at an absorption of 536 nm in a DU series 70 Beckman

spectrophotometer (Beckman Instruments Inc., Beaconsfield, U.K.). The amount of lipid was calculated through linear regression of the standard concentrations. The triplicates in each of the standard batches should show reasonable precision, recommended to be % CV of 5 % or less. (% CV is the standard deviation divided by the mean multiplied by 100).

### **3.3.3. High performance liquid chromatography analysis of clavulanic acid**

The aim of this assay was to analyse the titre of clavulanic acid accurately using high performance liquid chromatography with reference to a standard of known concentration, supplied by SmithKline Beecham. The assay was based on a method developed by Foulstone and Reading (1982).

#### **3.3.3.1. Equipment**

The isocratic high performance liquid chromatography assay for clavulanic acid was carried out through a Waters  $\mu$ bondapak C18 radial compression cartridge (11cm) and guard column. The pump used was a Waters 510 model HPLC pump. Absorbance was detected on a Waters 486 tuneable detector and results integrated and recorded on a Waters 746 data module (Waters Associates, Watford, U.K.). The samples were injected from a Waters 710B Wisp autosampler.

#### **3.3.3.2. Standard preparation**

The standard used was clavulanic acid lithium salt, 95.5% pure supplied by SmithKline Beecham Pharmaceuticals. This was stored in a refrigerator at 4°C in a jar containing Silica gel. To prepare the standard the salt was taken out of the refrigerator and allowed to warm to room temperature. 0.045 g of the standard was weighed on a Satorious four decimal place balance and made up to 10 mL in a volumetric flask with Milli Q water. The standard solution is stable stored for one month at - 40°C and 7 days at -20°C.

#### **3.3.3.3. Sample preparation**

10  $\mu$ L of HPLC grade glacial acetic acid was added to 2 mL of shaken broth in a 2.2 mL Eppendorf tube. This was then centrifuged on a bench top centrifuge at high speed for 10 minutes. The sample was then filtered through a 0.2  $\mu$ m syringe filter into a 1 mL neckless HPLC vials with plug caps (Chromacol, W.G.C., Hertfordshire, U.K.).

#### 3.3.3.4. Running buffer preparation

4.625 g of HPLC grade ammonium acetate was dissolved in 1 L of milli Q water. This was adjusted to pH 6.4 with HPLC grade glacial acetic acid and filtered through a 0.45  $\mu\text{m}$  filter into a 2 L Buchner flask. 2 mL of HPLC grade glacial acetic acid and 20 mL of HPLC grade methanol was added. Before use the buffer was sonicated for 25-30 minutes to remove air bubbles, and the top covered with parafilm.

#### 3.3.3.5. HPLC set-up

Running conditions:

Column:	Waters $\mu$ bondapack C18 radial compression cartridge
Buffer:	1500 mL of 60 mM Ammonium acetate, (pH 6.4), 3 mL HPLC glacial acetic acid and 30 mL HPLC methanol.
Flow rate:	3 mL min <sup>-1</sup>
Wavelength:	230 nm.
Run Time:	4 mins

The system was equilibrated by washing with running buffer at a rate of 1 mL min<sup>-1</sup> and gradually increased to 3 mL min<sup>-1</sup> for at least 15 minutes. A standard was run at the beginning and the end of a sample set. Duplicate injections were carried out on each sample. The retention time for the clavulanic acid peak is approximately 2.5 minutes.

#### **3.3.4. Ammonia assay**

This assay was based on the reductive amination of the sample with an alkaline nitroprusside solution to form a pale yellow complex, the absorption of which can be read at 625 nm.

##### 3.3.4.1. Reagents

*3.3.4.1.a. Phenol nitroprusside.* 15.5 g of phenol was added to 0.0625 g of sodium nitroprusside and made up to 500 mL with deionised water in a 500 mL volumetric flask. This was then divided into approximately 25 mL aliquots and stored in the freezer. Aliquots were defrosted when required.

*3.3.4.1.b. Alkaline hypochlorite.* 5.0 g of sodium hydroxide pellets were added to 5.38 mL of sodium hypochlorite solution, of spectrometric grade, in a 500 mL volumetric

flask. The solution was made up to 500 mL with deionised water. This solution was stored in the laboratory refrigerator.

**3.3.4.1.c. Ammonium standard.** A solution of  $1 \text{ g L}^{-1}$  of ammonium sulphate was made up with distilled water in a 1 L volumetric flask. 100 mL, 50 mL and 10 mL of standard solutions were made up to 2mL with deionised water and the assay procedure 3.3.4.2. followed. A standard curve was produced from which the ammonia in the samples could be calculated.

#### 3.3.4.2. Assay procedure

Whole broth samples were centrifuged in a bench top centrifuge on high for 10 minutes and filtered through  $0.2 \mu\text{m}$  Whatman syringe filter.  $10 \mu\text{L}$  of sample was made up to 2mL with deionised water and placed in a water bath at  $37^{\circ}\text{C}$ . After 1.5 minutes 1 mL of phenol reagent was added. 4 minutes after the samples were placed in the water bath 2mL of alkaline hypochlorite reagent was added. The samples were left for a further 28.5 minutes. The absorbance was read at 625 nm on a DU series 70 Beckman spectrophotometer. A blank of 2 mL deionised water was prepared with the samples to zero the spectrophotometer.

### **3.3.5. Phosphate assay**

This assay is based on inorganic phosphates reacting with the molybdate to form a stable yellow coloured complex. This complex then reacts with the ascorbic acid to form a blue/green colour, the absorbance of which can be read at 700 nm.

#### 3.3.5.1. Reagents

**3.3.5.1.a. Ammonium molybdenate:** 2.5 g of ammonium molybdenate was dissolved in 100 mL of water in a 100 mL volumetric flask. This was stored at room temperature.

**3.3.5.1.b. 10% Ascorbic acid:** 2.5 g of ascorbic acid was dissolved in 25 mL of water. This was stored at room temperature.

**3.3.5.1.c. Phosphate reagent:** The phosphate reagent solution was prepared by adding 1 volume of 6 N sulphuric acid, 2 volumes water and 1 volume of each ascorbic acid

and ammonium molybdenate reagents. This reagent was prepared fresh for each set of samples.

#### 3.3.5.2. De-proteinisation

Before the phosphate assay was carried out the whole broth samples were centrifuged with an equal volume of 20% tricarboxylic acid (T.C.A.). This was carried out to remove proteins which cause precipitation in the phosphate assay.

#### 3.3.5.3. Standard preparation

Standard solutions for the phosphate assay were prepared from potassium dihydrogen orthophosphate in water to concentrations of 2.0, 1.5, 1.0, 0.5, and 0.0 g L<sup>-1</sup>.

#### 3.3.5.4. Assay

100 µL of deproteinised sample, or standard, was placed in a capped Eppendorf tube. To the sample was added 350µL of freshly prepared phosphate assay reagent. The tubes were capped and incubated in a water bath at 37°C for 15 minutes. The absorbance of the samples was read at 700 nm on a DU series 70 Beckman spectrophotometer.

### **3.4. Analysis of fatty acids by gas chromatography - Method development**

Due to the variability in the results obtained from the total lipid analysis using the spectrophotometric vanillin assay a more accurate technique of detecting lipids in biological material was needed. The core analytical techniques for lipids have remained constant over the past few decades and include organic extraction, thin layer chromatography (TLC) gas chromatography (GC) high performance liquid chromatography and gas chromatography linked with mass spectrometry (Bausch, 1993). Analytical methods other than chromatography and/or mass spectrometry are of little use for accurate resolution and identification of lipid species even in simple mixtures. General strategies in chromatographic analysis of lipid classes have been reviewed by Myher and Kuksis (1995) and Hoving (1995). The total lipid assay is also unable to distinguish between classes of lipid, such as tri-, di-, and monoacylglyceride, free fatty acids or phospholipids contained within the cellular membranes. A gas

chromatography method for the analysis of fatty acids in fermentation broth was developed, adapted from a previous method described by Lepage and Roy (1986).

### **3.4.1. Equipment**

Fatty acid and fatty acid methyl ester (F.A.M.E.) analysis was carried out through a 0.25 mm Stabilwax<sup>®</sup> - DA 30 m capillary column with an internal diameter of 0.53 mm (supplied by Thames chromatography, Windsor, Berkshire, U.K.). Stabilwax-DA columns contain a bonded polyethylene glycol stationary phase that is specifically deactivated to allow the analysis of acidic compounds without derivatisation. The deactivation reduces adsorption to the stationary phase and may increase the sample capacity of volatile free fatty acids. Stabilwax-DA allows the free fatty acids, ranging from C1 to C20, to be analysed without derivatisation.

### **3.4.2. Free Fatty Acid Analysis**

A mixture of free saturated and unsaturated fatty acid standards ranging from a chain length of 6 to 18 carbon units (supplied by Sigma) were dissolved in chromatography grade ethanol. The GC method used to analyse the standards was adapted from the column manufacturers recommendations. The column was initially held at 100°C for 2 minutes. The temperature was increased at a gradient of 8°C per minute until the final temperature of 250°C was reached. The column was then held at 250°C for a further 5 minutes. The total run time was 26 minutes. Peak resolution for a mixed sample of free fatty acid standards was good with reproducibility of 5.0-7.0 % relative standard deviation (RSD).

A standard rapeseed oil solution was made up in chromatography grade ethanol and analysed using the same GC program as for the standards. Resolution of free fatty acids in rapeseed oil was good using the method described. However, the column detected approximately 30% of initial concentration loading.

Analysis of a selection standard triacylglycerides and saturated free fatty acid combinations was carried out using the same gas chromatography method as above. Analysis of standard triacylglycerides, ranging from 8 to 16 carbon units, was not possible using the column selected as no peaks were detected. When in combination with a free fatty acid of the same carbon unit length, only the free fatty acid was



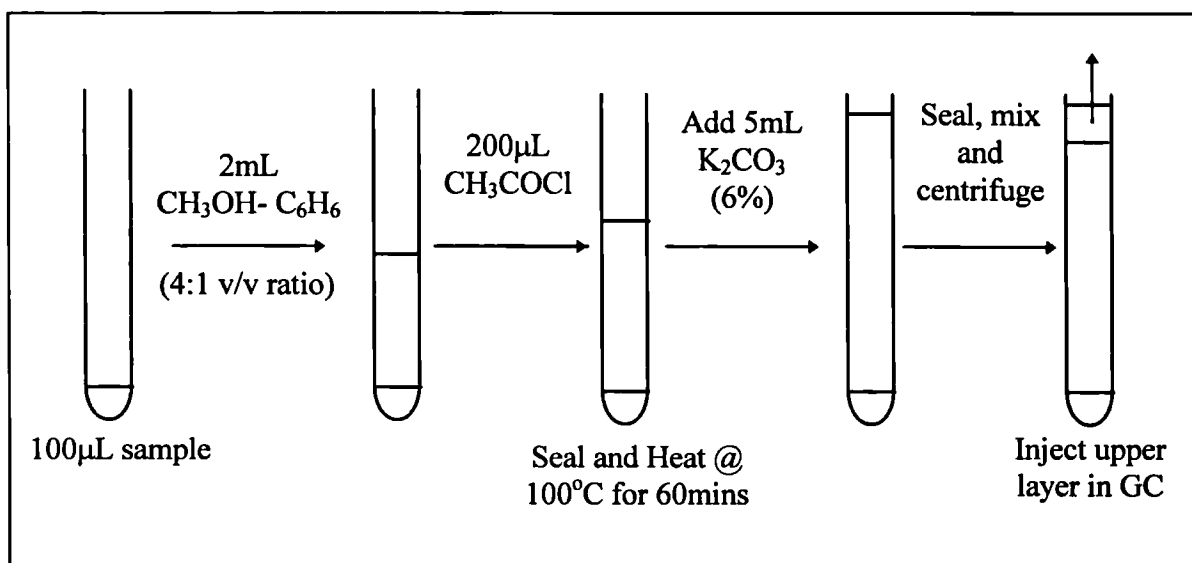
detected. The low level of fatty acid detection in the rapeseed oil analysis may be due to the complex composition of the rapeseed oil. Derivatisation of the fatty acid will result in higher resolution and may increase recovery efficiencies.

### **3.4.3. Derivatisation of free fatty acids**

Derivatisation changes the volatility of the lipid components and will improve peak shape, thus providing better separation. The majority of derivatisation procedures involve conversion of the fatty acid components of the lipid into the corresponding esters, usually methyl esters. The reaction is referred to as transesterification because the reaction involves cleavage of an ester by an alcohol. Methods of fatty acid methyl ester (F.A.M.E.) preparation vary considerably in terms of the number of steps involved, the solvents and reagents used, conditions applied and sample volumes tested. The preparation of F.A.M.E.'s for gas chromatography in the analysis of lipids in biological materials has been reviewed by Liu (1994).

The method used for F.A.M.E. preparation from rapeseed oil was a one hour direct transesterification procedure initially developed by Lepage and Roy (1986). The methylation is carried out with acetyl chloride in the presence of a solution methanol:benzene. Recoveries were reported to be greater than 95% with highly reproducible results. A summary of the procedure devised by Lepage and Roy (1986) is shown in Figure 3.3.

The method involved adding 2 mL of a methanol:benzene solution, 4:1 (v/v), to 100 $\mu$ L of sample. 200  $\mu$ L of acetyl chloride was added slowly over a period of a minute. Heating the sample and reagents was carried out in an oven set at 100°C for 60 minutes. This was required to speed up the reaction. 6% (w/v) K<sub>2</sub>CO<sub>3</sub> solution was added to terminate the reaction and neutralise the mixture. The potassium solution was added slowly due to the release of CO<sub>2</sub>. The tubes were then shaken and centrifuged, and an aliquot of the benzene upper layer injected into the GC. The methylation was carried out in glass tubes with sealed metal screw caps.



**Figure 3.3. Schematic diagram of the methylation procedure for a lipid sample.**

**(Lepage and Roy, 1986).**

#### **3.4.4. Method development**

Using the method described by Lepage and Roy (1986) detection of fatty acids in fermentation media had increased from 30% to 60%. The initial sample volume was found inadequate for fermentation broth through reproducibility studies due to the nature of the lipid in the fermentation media. Sample volume was increased. As sample volume was increased the volume and ratio of methanol:benzene mix, and amount of acetyl chloride added was increased. The ratio of methanol to benzene was decreased from 4:1 to 2:1 and the volume added increased due to problems in the solubility of the rapeseed oil. Due to incomplete methylation of the fatty acids, noted by the presence of unmethylated free fatty acid peaks, the volume of acetyl chloride was also increased. Acetyl chloride is highly reactive with water and therefore too large a volume of acetyl chloride was difficult to get into solution. The final method was set at 500  $\mu$ L of sample methylated using the acid catalysed method with 1 mL of acetyl chloride in the presence of 15 mL of a 2:1 (v/v) ratio methanol:benzene solution.

The reaction was terminated with 5 mL of 6% (w/v)  $K_2CO_3$ . The upper benzene layer was not large or clear enough to be injected directly onto the GC column. The methyl esters were therefore extracted into hexane. The efficiency of recovery increased to approximately 80-85%.

The method of extracting the methyl ester from the reaction was investigated. Initial extraction was carried out using 5 mL hexane with injection of the upper layer. The hexane extraction was repeated and the layer injected separately. Combining two hexane extractions increased the efficiency of recovery to approximately 95% with a sample RSD of 5.0%. A third extraction did not increase efficiency significantly and was considered unnecessary. The results for total lipid analysis and gas chromatography of F.A.M.E.'s showed good correlation.

### **3.5. Particle size distribution analysis**

#### **3.5.1. Continuous and dispersed phases**

The continuous phase was distilled water containing 0.3% SDS. The dispersion phase was Flour 1 or Flour 2.

#### **3.5.2. Sample analysis equipment**

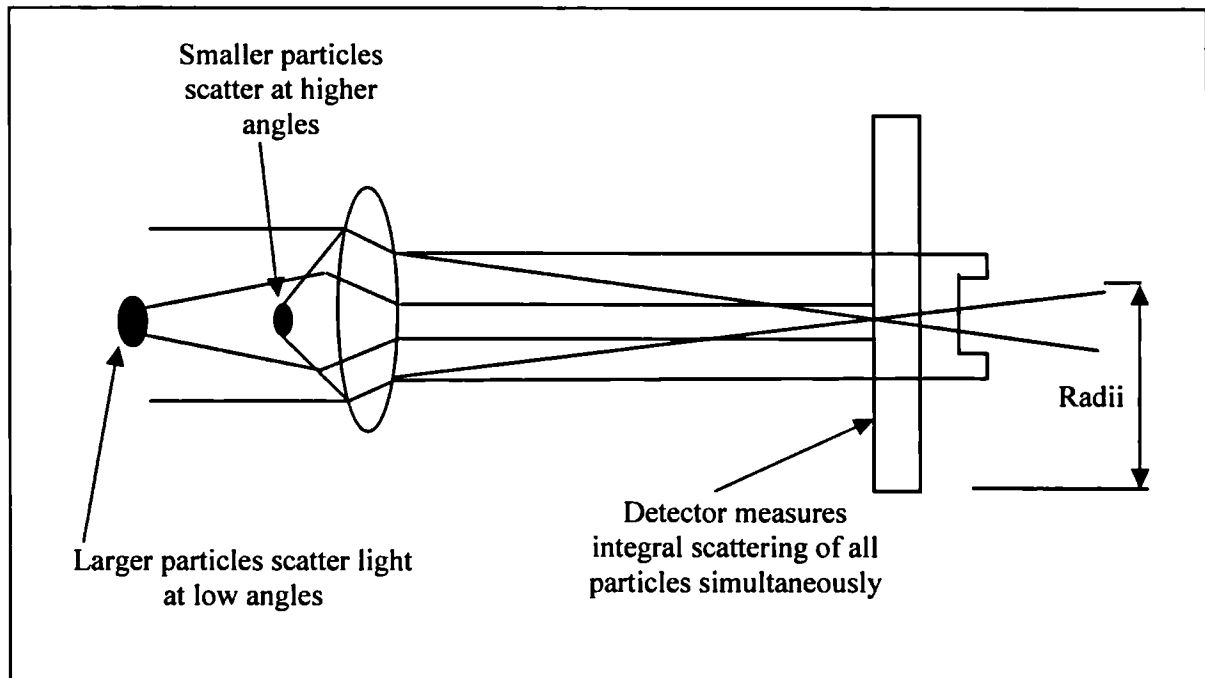
The particle size of the dispersion phase was measured using a 3600Ec Malvern particle sizer (Malvern Instruments, Malvern, U.K.) The instrument uses laser diffraction to measure the size of particles. This is done using the principle of the Fraunhofer theory in measuring and interpreting angular distribution of light diffracted by the droplets/particles.

#### **3.5.3. Operation of Malvern laser equipment**

The Malvern laser equipment uses a low power laser transmitter to produce a monochromatic beam of light that illuminates the sample droplets or particles flowing in an appropriate cell. The illuminated particles then diffract the incident light. The degree of diffraction is inversely related to the size of the particles. The principle of laser diffraction is illustrated in Figure 3.5.

A continuous flux of particles through the illuminated area is then collected on a series of concentric detection rings and integrated over a suitable period of time, giving a diffraction pattern that is representative of the bulk sample. An analogue signal proportional to the incident light intensity is produced by the Fourier transform lens focusing the diffraction pattern onto a multi-element photoelectric detector. This detector is linked to a computer which reads the diffraction pattern.

Having measured the diffraction pattern the computer uses non-linear least squares analysis to find the size distribution which gives the most closely fitting diffraction pattern. The computer produces 31 channels of data which are then presented on a volume basis. The machine is able to measure particles within the range of 5  $\mu\text{m}$  up to 564  $\mu\text{m}$  in diameter.



**Figure 3.4. The principle of laser diffraction.**

#### 3.5.4. Sample analysis procedure

The particle size measurements were performed using the Malvern Laser equipment and associated software. The cell was then filled slowly, so as to prevent the formation of bubbles, with distilled water to the required level and placed in the machine. Using a focal length of 300nm, the laser was then aligned to ensure even distribution of the light and to maximise light intensity. The laser light illuminated the central region of the cell above the detector port and the magnetic stirrer switched on. The buffer intensity was then measured. The reading was in the upper region of the scale on the screen (GOOD) and the output channel should read LOW. Once a steady background was obtained the sample was added as fine droplets to ensure complete mixing. The sample was added until concentrated enough to give an obscuration reading of approximately 0.2. The readings were then taken and analysed as percentage volume in the size band.

Intensity is a function of particle size and concentration and therefore the sample concentration limits depend upon the size of the material. At lower concentrations, poor signal level and large random error exist whilst at the higher concentrations multiple effects may produce systematic error. In all analysis performed the lens focal length was set at 300 mm. Particles in the size range from 6 to 350  $\mu\text{m}$  were detected with an accuracy of approximately 4% on the basis of volume.

### **3.6. Image analysis**

Morphological characterisation was carried out on fermentation broth of *S. clavuligerus* using the image analysis technique described by Packer and Thomas (1990).

#### **3.6.1. Sample preparation**

Morphological characterisation was carried out on dry slides prepared immediately after fermenter sampling. The slide needed a high enough concentration of hyphae in order to ensure a reasonable number of objects to be measured in a single field. If the concentration were too low the time analysing each field would be excessive and if the concentration were too high hyphal overlap would interfere with measurement. A dilution of x400 in T4 Ringers solution (pH 7.0) was found sufficient for all samples. A 40  $\mu\text{L}$  aliquot of sample was spread over a glass microscope slide and left to air dry for 24 h. The samples were stained by submersion for 2 minutes in a methylene blue solution (0.3 g methylene blue, 30 mL 95% ethyl alcohol, in 100 mL deionised water) washed with deionised water and finally air dried for a further 24 h. Triplicate slides of each sample were prepared.

#### **3.6.2. Image analysis equipment**

For image analysis a Magiscan 2A image analyser (Joyce Leobl Ltd, Gateshead, U.K.) running GENIAS image analysis software was used. This was attached to a Polyvar microscope (Reichert Jung, Vienna, Austria) with x25 objective set for bright field illumination and an automatic stage giving automatic x and y motion. For each sample 10 x 10 fields were analysed in order to analyse at least 300 objects from each fermentation sample.

In each field the image was captured and digitised to a representation consisting of 512 x 512 pixels and 255 shades of grey, 0 being black and 255 being white. Pixels above a designated threshold were ignored, so analysis was carried out only on dark objects, that is, hyphae and broth debris. The GENIAS software allowed a number of actions to be carried out on the grey image to enable the dimensions of the hyphae to be calculated.

Objects other than hyphal, i.e. undissolved solids present in the media, were distinguished from the hyphae by their size and shape. A circularity parameter (Packer, 1991) removed particulate material as it depends on the circularity of the object:

$$\text{Circularity} = 4 \times \pi \times \text{total area} / (\text{perimeter})^2$$

Circularity equals one for a closed circle and closer to zero for a long thin fibre. It was found that objects with a circularity of greater than 0.4 are undesirable objects in the case of *S. clavuligerus* (Packer and Thomas, 1990) and therefore any objects with a circularity of greater than 0.4 were excluded.

### 3.6.3. Morphological measurements

Table 3.8. describes the main morphological measurements used in image analysis of each field of fermentation samples.

**Table 3.8. Description of morphological parameters used in image analysis.**

Parameter	Abbreviation	Description
Hyphal length	HL	Total length of all hyphae in an individual filament i.e. HL = ML + BL
Main hyphal length	ML	Length of the longest unbranched hyphae
Branch length	BL	Length of all branches from main hyphae
Filament area	MA	Total area occupied by individual filaments
Number of tips	NT	Total number of tips on an individual filament
Total clump area	CA	Total area occupied by an individual clump. A clump is defined as any object containing a single closed area.

The study suffered from elimination of larger hyphae due to the definition of a clump. Any object containing an enclosed area is classified as a clump. If an object did not contain an enclosed area it was considered as free mycelia. Larger hyphae are more likely to contain an enclosed area and are therefore measured as clumps. The best way to separate larger hyphae from clumps would be to redefine a clump as an object containing greater than three enclosed areas. Unfortunately a program with this function was not available at UCL.

#### **4. THE EFFECT OF MEDIA COMPOSITION AND pH ON LIPID UTILISATION IN *S. CLAVULIGERUS***

There are many parameters to consider when investigating the effects of process variables on lipid utilisation and production of clavulanic acid in 5 L batch fermentation of *S. clavuligerus*. Control of pH is a crucial parameter in a batch fermentation due to well known effects of pH changes on growth and enzyme activities. pH is a major economic concern in an industrial process in terms of optimising productivity as clavulanic acid itself is only stable between a pH of 6.5 and 7.2 (Tarbuck *et al.*, 1985). 5 L batch fermentations were carried out to investigate the effect of control of pH on growth, lipase activity and lipid utilisation. pH was controlled in base direction only as this is carried out in the production process. Clavulanic acid assays were not carried out on the pH comparison fermentations due to development of the HPLC technique used to quantify product titres.

Design of media composition is also critical in terms of optimising process productivity. Due to the economic significance of clavulanic acid little manufacturing process information has been published relating to media used on production scale. Pilot plant scale media has been described in various patents (British patent No. 1,563,103, Japanese patents 53-104796 and 55-162993). All patents show that the most important nutrients with respect to clavulanic acid production are soyabean protein, a high carbon sugar source and addition of rapeseed oil. As oil has been shown to increase antibiotic titres, Section 2.4.3., initial 5 L batch fermentations were carried out to investigate the need for rapeseed oil as a carbon source for increased, and extent of, productivity in the production strain of *S. clavuligerus*. Ratios of carbohydrate to oil carbon sources were also investigated with regard to the effect on lipid utilisation, lipase activity and clavulanic acid titres. Nitrogen source also plays a crucial role in the control and productivity of a bioprocess. Effects of variations in soya flour concentration and type, with respect to particle size, on lipase activity and lipid utilisation were also investigated.

As the residual oil level may be due to incomplete metabolism of components of rapeseed oil the effects of addition of some of the individual triacylglycerides present in the oil and their respective methyl esters were investigated in 2 L shake flask



experiments using complex media. These fermentations were carried out in order to identify any preferential utilisation of the constituents of rapeseed oil and/or any associated toxicity.

#### **4.1. pH Control**

Control of pH is a critical operation within a batch fermentation due to the effects<sup>of</sup> pH on growth and enzyme activity. Previous 5 L *S. clavuligerus* batch fermentations carried out by Cavanagh (1996) contained complex media with additional phosphate buffer. Addition of a buffer is not practical on a large scale. The production process is controlled in a base only direction and therefore the control of the pH of a fermentation and its effects on growth, lipase activity and lipid utilisation were investigated on a 5 L pilot plant scale.

pH comparison fermentations were carried out in the 7 L series 210 LH fermenter with a 5 L working volume, Figure 3.1. Process parameters were as described in Table 3.5. Dissolved oxygen tension was maintained above approximately 10% by increasing agitation rates. pH was maintained above pH 7.0 with the closed loop controlled addition of a 30% ammonia solution. Process media was as described in Section 3.1.9. Soyaflour concentrations used are given in concentrations relative to the concentration of modified starch, i.e. 3:1 indicates three parts flour to one part modified starch.

##### **4.1.1. The effect of pH control on growth**

Due to the particulate nature of the complex media and the hyphal network of *S. clavuligerus* traditional methods of biomass quantitation, such as dry weight and optical density, cannot be used. Total protein measurements cannot be carried out as a measure of change in biomass due to the presence of protein in the soyaflour constituent of the complex media. The effect of pH on growth was measured using on-line dissolved oxygen tension levels. Exit gas analysis, oxygen uptake rates and carbon dioxide evolution rates, were also reported where possible.

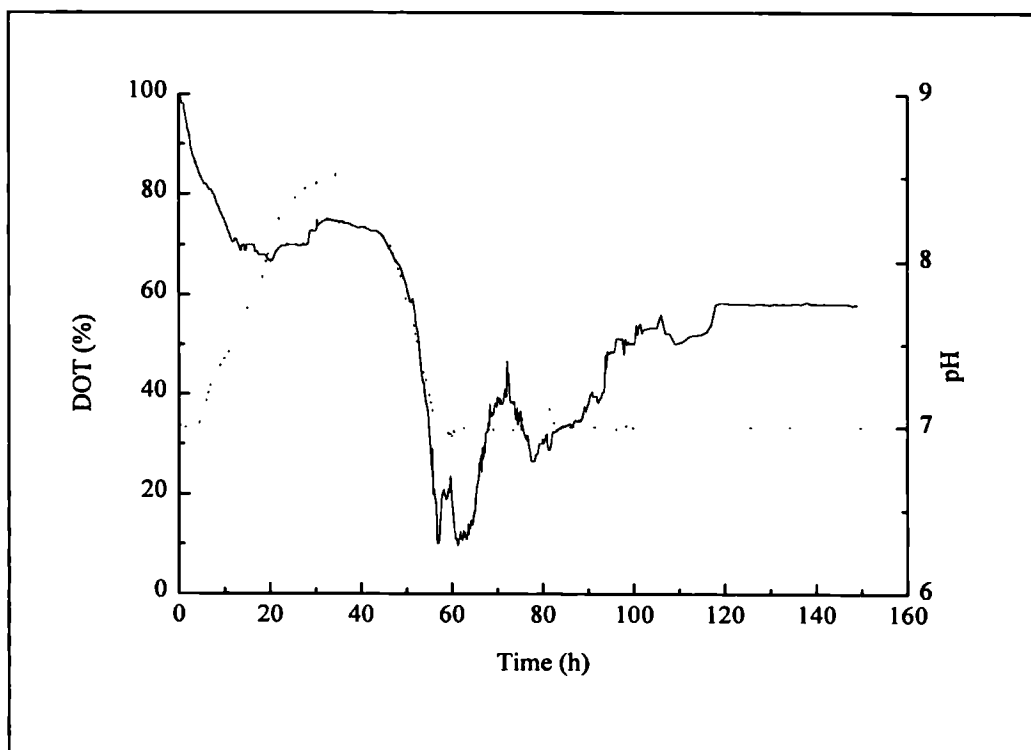
##### **4.1.1.1. Dissolved oxygen tension (D.O.T.)**

Cells in aerobic culture take up oxygen from the surrounding media. Rates of oxygen transfer are therefore of prime importance when analysing fermentation data, especially

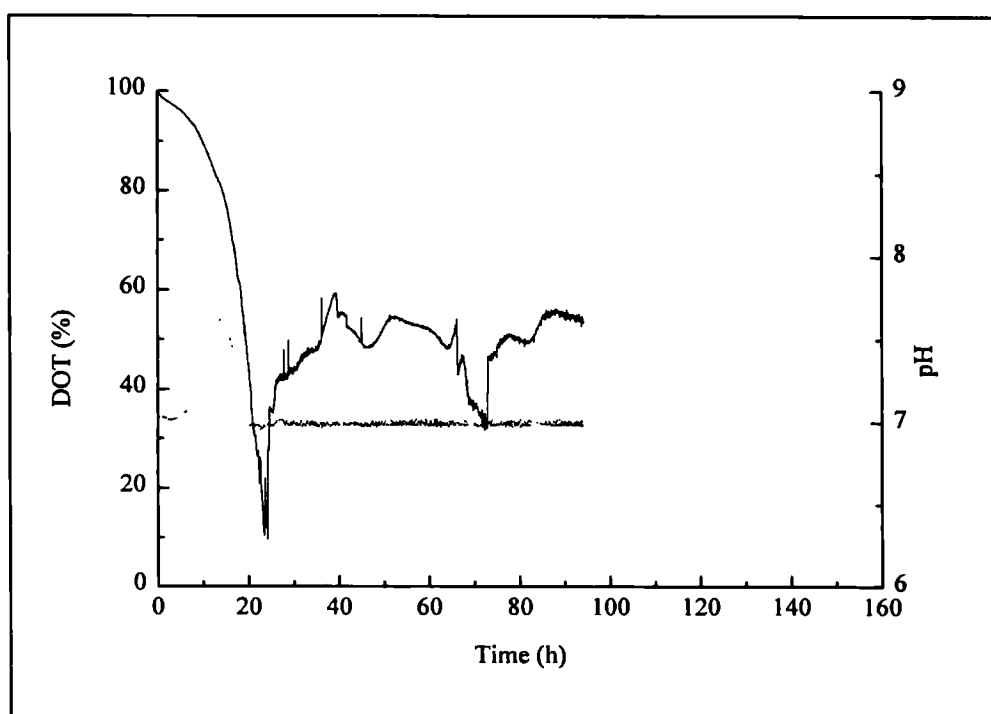
at high cell densities when cell growth is likely to be limited by availability of oxygen in the process. Measurement of changes in percentage DOT give an indication of cell activity and can be related to changes in cell growth. Levels of dissolved oxygen tension, and therefore cell growth, during a fermentation process are influenced by changes in both the physical and chemical characteristics of the surrounding environment. The effects of changes in pH and the method of pH control on DOT were investigated on a 5 L scale.

Figure 4.1.a. shows the DOT profile and pH changes in a base only controlled fermentation containing 3:1 soyaflour:modified starch. Initially, 0-15 h, the DOT decreased as the organism takes up oxygen due to cell growth. No significant lag phase was observed which indicates correct seed transfer. After 15 h the DOT was maintained at approximately 70%. A constant DOT indicates a steady state as cell oxygen uptake rate is equal to oxygen transfer rate into solution. At this time the pH increased above pH 8. A maximum of pH 8.5 was reached at 35 h. After 50 h the pH fell below 8 and the DOT decreased, indicating a restoration in cell growth. pH and DOT continued to decrease until 60 h, after which base addition maintained the pH at the set point of 7.0. The agitation rate was increased at 56 h from 625 rpm to 725 rpm, and at 60 h to 825 rpm, in order to maintain the DOT above 10%. When pH is maintained at 7.0 DOT increased indicating the stationary phase of cell growth. A second decrease in DOT was noted at 80 h and correlates with a second increase in pH.

Figure 4.1.b. shows the DOT profile and the changes in pH in a base only controlled fermentation containing 2:1 soyaflour:modified starch. Initial growth rates for the fermentation containing 2:1 soyaflour were slower than the fermentation containing 3:1. This difference may be due to differences in seed transfer although the initial shake flasks were inoculated with the same number of spores and incubated for the same length of time. There was not a significant difference in DOT levels taking into account fermentation reproducibility. The following decrease in growth, represented by the continuous fall in DOT, was due to the pH being maintained below a level which does not significantly affect the growth of the organism. Agitation rate was increased at 22 h from 625 rpm to 825 rpm to maintain the DOT above 10%. As seen in the fermentation containing 3:1 soyaflour there is a further decrease in DOT at approximately 70 h, but no observed change in pH.

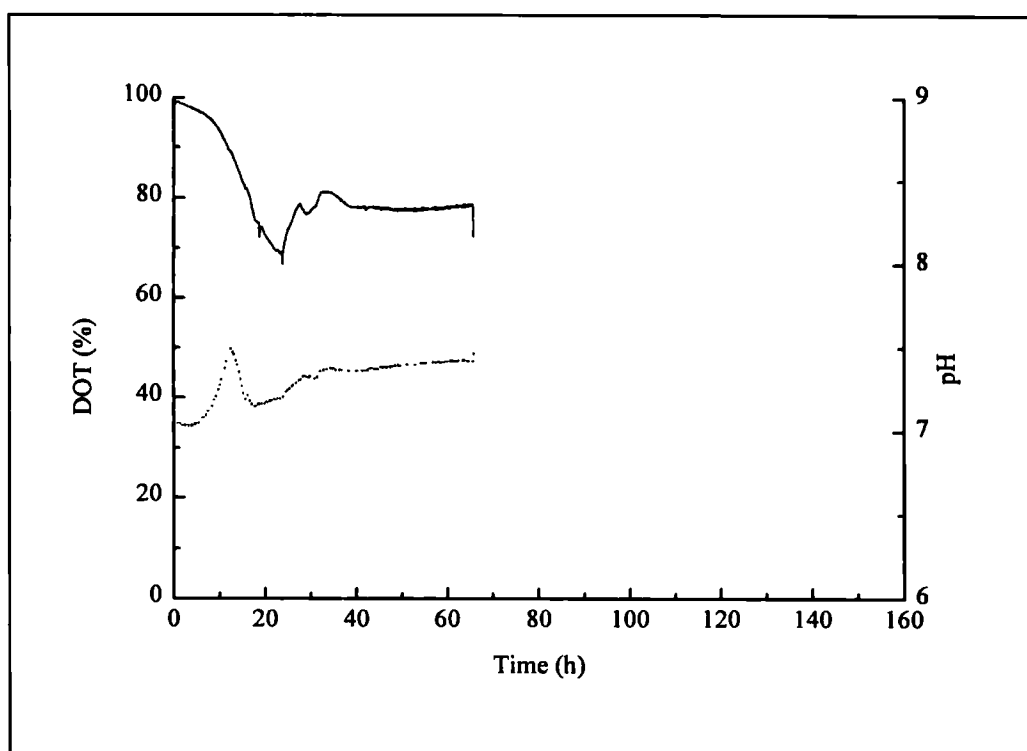


**Figure 4.1.a. DOT ( - ) and pH ( - - ) profiles for the base only controlled fermentation containing 3:1 soyaflour**



**Figure 4.1.b. DOT ( - ) and pH ( - - ) profiles for the base only controlled fermentation containing 2:1 soyaflour**

DOT profile in Figure 4.1.c. showed that when the level of soyaflour was reduced to 1:1 the initial fall in dissolved oxygen was more gradual than in previous fermentations. After 24 h the dissolved oxygen tension increased indicating growth had ceased. This indicates that 1:1 soyaflour delivers an insufficient level of nutrients for the maintenance of growth in a 5 L batch fermentation of *S. clavuligerus* in complex media. Fermentation was terminated after 67 h.



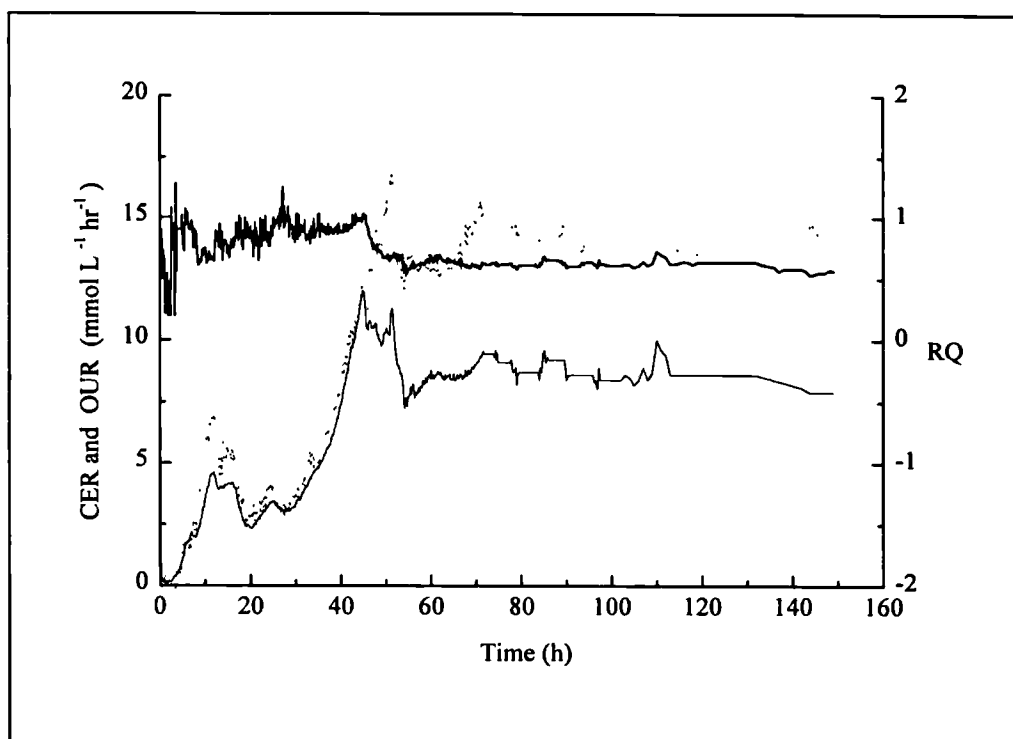
**Figure 4.1.c. DOT ( - ) and pH ( - - ) profiles for the base only controlled fermentation containing 1:1 soyaflour**

#### 4.1.1.2. Exit gas analysis

Oxygen uptake rate, OUR, and carbon dioxide evolution rate, CER, Figure 4.2., show the pattern of growth during the base only pH controlled fermentation containing 3:1 soyaflour. Data confirmed that the rise in pH above 8.0 decreased the rate of growth of *S. clavuligerus* in 5 L batch fermentation. Growth was then restored as the pH fell below 8.0.

The respiratory quotient (RQ) value for growth on a carbohydrate source is approximately 1.0-0.9 and the value for growth on a lipid is approximately 0.7-0.6. The switch from growth on the carbohydrate, modified starch, as the main carbon source, to

the use of rapeseed oil was clearly seen after approximately 40 h. The switch in RQ corresponds with the initiation of a stationary phase of growth. Exit gas analysis data was not available for the base only controlled fermentations containing 2:1 and 1:1 soyaflour due to equipment and data logging failure.



**Figure 4.2. Exit gas analysis for a base only controlled fermentation containing 3:1 soyaflour. ( --- OUR — CER — RQ )**

A decrease in initial pH rise correlated to a decrease in the initial soyaflour content of the complex media. The rise and fall in pH during the process may be due to the level of ammonium ions that are released from the metabolism of proteins present in the soyaflour component of the process media. A decrease in soyaflour concentration decreased the ammonia levels detected in the broth, Figure 4.3. The ammonia profile for the fermentation containing 3:1 soyaflour corresponds to the pH profile for that fermentation, Figure 4.1.a. Metabolism of the soyaflour component of the complex media releases ammonium ions that may be responsible for the pH changes during the process.

There are many factors that could contribute to the decrease in pH of the fermentation media including the decrease in the concentration of ammonium ions, through metabolism, and the increase in the concentration of fatty acids from the metabolism of

the oil component of the media. Carbon dioxide released from respiration may also contribute to decreasing pH.

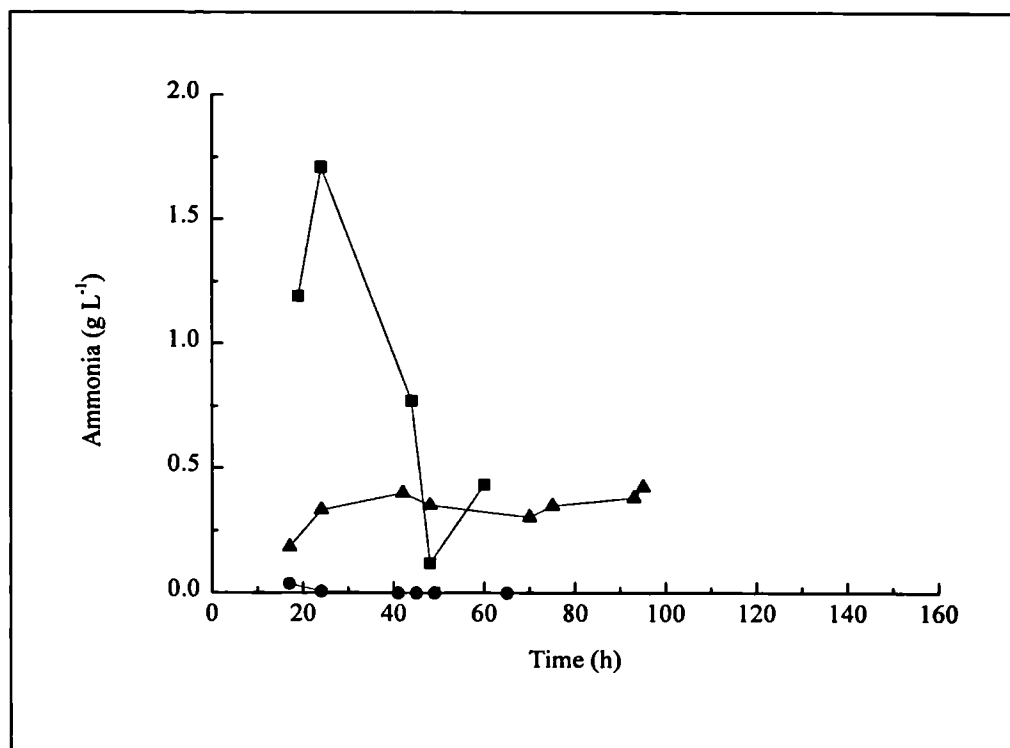


Figure 4.3. Ammonia profiles for base only controlled fermentations containing 3:1 (■), 2:1 (▲) and 1:1 (●) soyaflour.

#### 4.1.2. Lipase activity

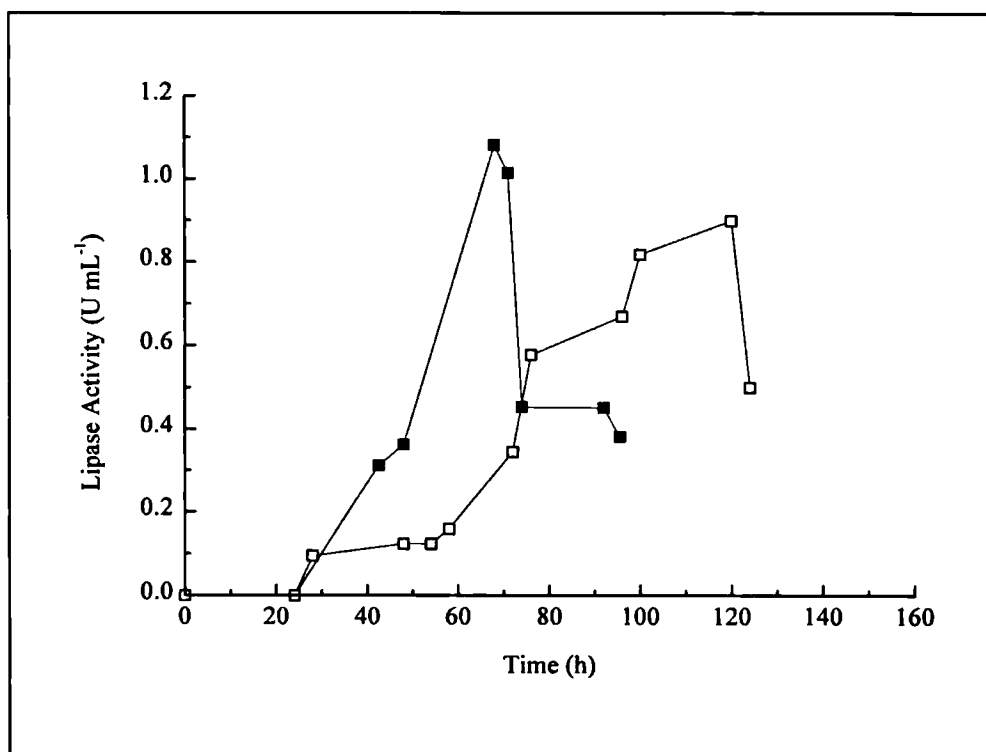
Lipase activity was measured by automatic titration against 0.01 M NaOH to maintain the reaction mixture at a set point of 7.2, using a tributyrin substrate. Results on development of an assay for lipase activity in *S. clavuligerus* 5 L batch fermentations (Cavanagh, 1996) concluded that this was the pH for maximum assay sensitivity. pH of a fermentation has a significant effect on the activity of many enzymes. Effect of pH control in a fermentation on lipase activity was investigated. Triplicates of each assay were performed and the mean of the results plotted.

Figure 4.4.a. compares the lipase activity detected throughout base only and full pH controlled fermentations containing 3:1 soyaflour. Lipase activity was not detected until 24 h into a fermentation. The delay in detected activity may suggest that lipase activity is not required during the initial stages of the fermentation process due to the initial metabolism of the carbohydrate carbon source, modified starch. Initial lipase activity may also be below the detection limit of the assay. When lipase activity in Figure 4.4.a.

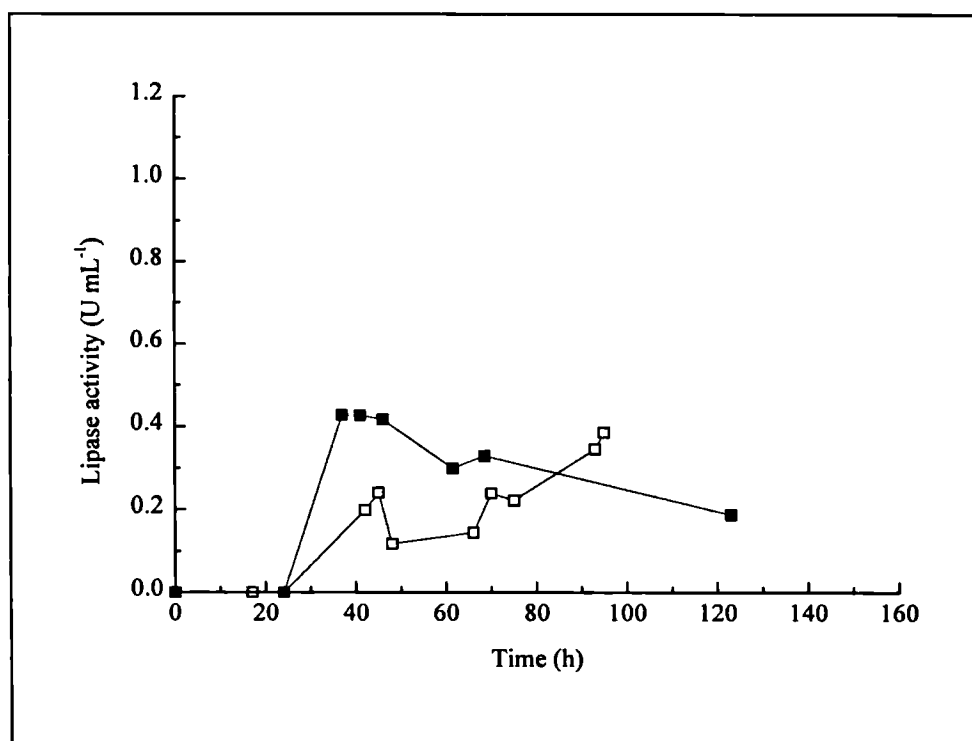
was compared to the pH changes in Figure 4.1.a., it can be seen that as the pH increased the lipase activity remained relatively low, until the pH was reduced to 7.5 at approximately 60 h. After 60 h lipase activity increased to a maximum of  $0.9 \text{ U mL}^{-1}$ . The maximum lipase activity in the fermentation controlled by both acid and base was higher,  $1.1 \text{ U mL}^{-1}$ . Maximum activity in the fully pH controlled process occurred 50 h before the maximum in the base only controlled fermentation. The increase in pH delayed detected lipase activity in the base only controlled fermentation. An increase in pH was also found to alter the growth rate of the organism during fermentation and therefore volumetric lipase activity is affected. A comparison of specific lipase activity would give a good comparison of actual lipase activities irrespective of growth rates. Unfortunately these data are not available due to methods of biomass quantitation used in the initial stages of the project.

Figure 4.4.b. shows the lipase activity in full pH and base only controlled fermentations containing 2:1 soyaflour. As with the fermentation containing 3:1 soyaflour there was a delay in detection of lipase activity of approximately 24 h. Initial rise in activity, 24 to 48 h, was higher in the pH controlled process than in that of the base only controlled fermentation. Lipase activity in the base only controlled fermentation took approximately 60 h longer to reach the same level of activity. Due to the control of pH below 7.5 in the base only controlled fermentation, growth in both processes was very similar. Specific lipase activity cannot be measured, but due to the similarities in growth rates, measured through D.O.T., volumetric lipase activity may be compared.

No lipase activity was detected in the base only controlled fermentation containing 1:1 soyaflour. The DOT profile showed that growth ceased after 30 h. As lipase activity was not detected until after 24 h in the previous fermentations containing more soyaflour no activity was expected.



**Figure 4.4.a. Lipase activity in pH controlled ( ■ ) and base only controlled ( □ ) fermentations containing 3:1 soyafLOUR.**



**Figure 4.4.b. Lipase activity in pH controlled ( ■ ) and base only controlled ( □ ) fermentations containing 2:1 soyafLOUR.**

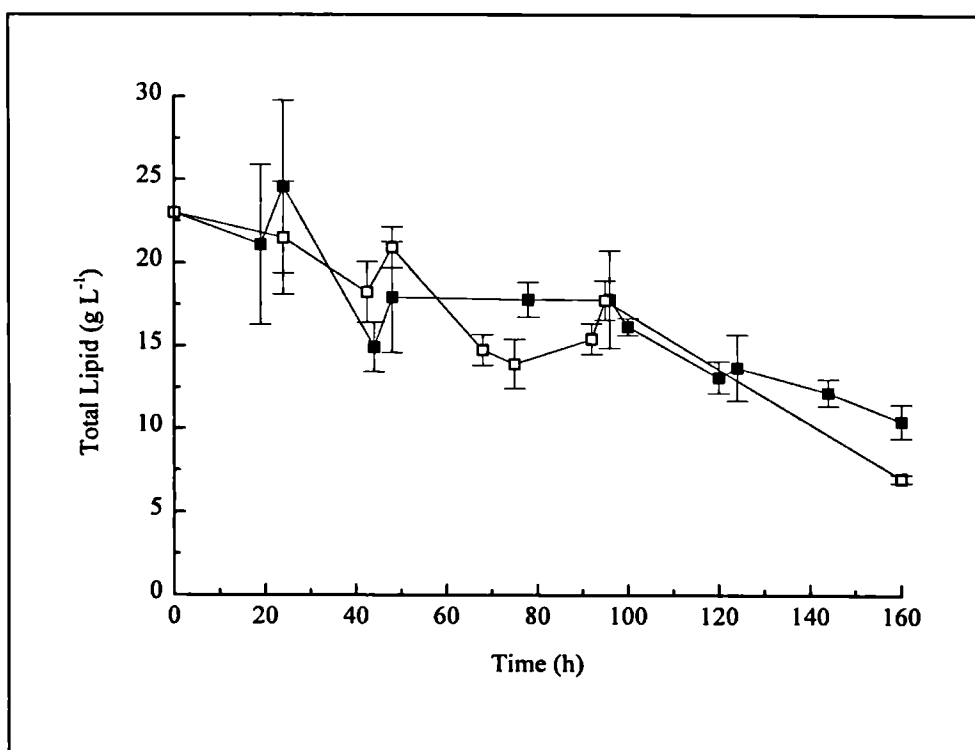


#### 4.1.3. Lipid utilisation

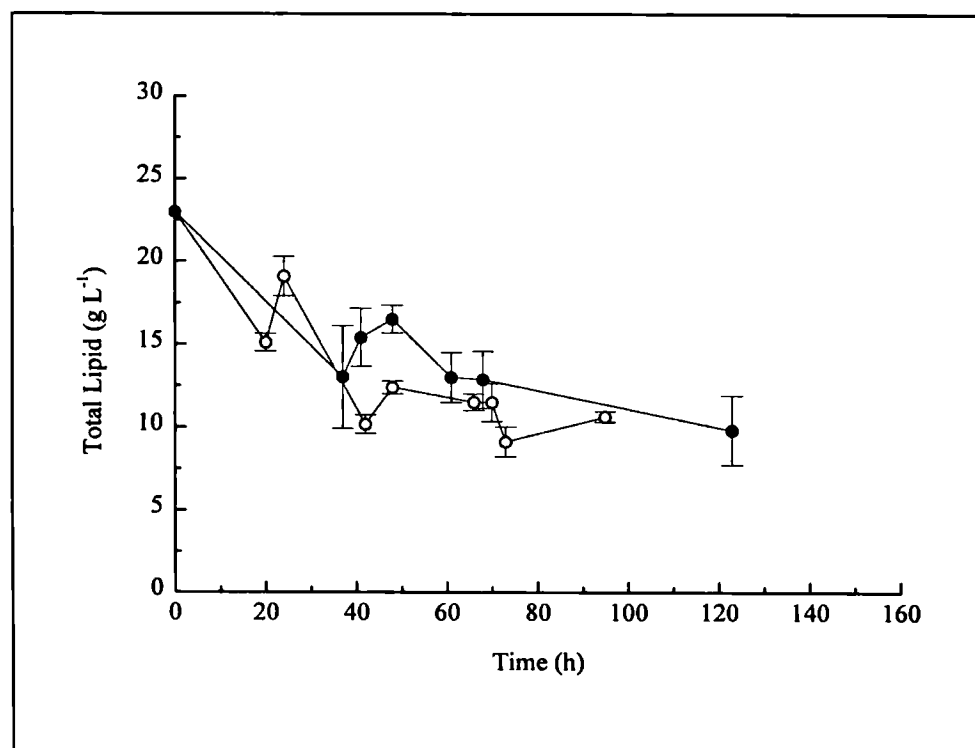
The vanillin method used for total lipid analysis, Section 3.3.2., showed variability in the results due to non-uniform dispersion of the lipid droplets in the fermentation broth analysed. Triplicates of each sample were assayed and the mean plotted. Variability of the assay within each replicate is indicated in the results as standard deviation from the mean by bars of error indicating a 67% confidence limit. Initial error bars were wide. This may be due to the larger oil droplet size as observed under the microscope. Towards the end of the fermentation total lipid analysis becomes less variable. This may be due to the lipid becoming more dispersed in the complex media and a decrease in the size of the oil droplets (Cavanagh *et al.*, 1994).

Figure 4.5.a. shows the rate of lipid utilisation and residual oil levels in pH controlled and base only controlled fermentations containing 3:1 soyaflour. Results indicate that there was no significant difference between the rates at which the rapeseed oil was utilised. A similar conclusion can be drawn from the comparison of pH controlled and base only controlled fermentations containing 2:1 soyaflour, Figure 4.5.b. Lipid utilisation seemed unaffected by increases in pH, although the lipase activity is significantly influenced. Only a basal level of lipase activity may be needed to be expressed in order to utilise the rapeseed oil in the process media.

The previously noted problem of residual oil (Stowell, 1987) is highlighted in Figures 4.5.a. and 4.5.b. The residual oil level was approximately 30-35% in all fermentations. Results conclude that the residual oil level is relatively unaffected by the changes in pH, growth and lipase activity.



**Figure 4.5.a. Lipid utilisation in pH controlled ( ■ ) and base only controlled ( □ ) fermentations containing 3:1 soyafLOUR.**



**Figure 4.5.b. Lipid utilisation in pH controlled ( ● ) and base only controlled ( ○ ) fermentations containing 2:1 soyafLOUR.**

#### 4.1.4. Discussion

pH of the fermentation broth affects the rate of growth of *S. clavuligerus* in a 5 L batch fermentation containing complex process media. A cessation in growth was observed above pH 8.0. The increase in pH in the base only controlled fermentations may be due to the release of ammonium ions from the metabolism of the soyaflour component of the complex media. If the concentration of flour is increased the ammonia concentration detected in the media increased. An increase in ammonia was correlated to the initial rise in pH.

Lipase activity was measured using an assay optimised by Cavanagh (1996). The titrimetric assay was optimised in terms of pH and substrate reagent. A tributyrin substrate was chosen in preference to rapeseed oil due to the ease of emulsification and assay reproducibility. Although using a tributyrin substrate does not give actual levels of activity present in the fermenter all assays were carried out using tributyrin for comparative activity results.

There was a delay of approximately 24 h in detection of lipase activity which may be due to detection limits of the assay or the initial utilisation of the modified starch carbon source in the complex media not requiring lipase activity. Lipase activity was greatly affected by changes in pH. Maximum lipase activity in pH controlled fermentations was higher and appeared earlier than in base only controlled fermentations. This may be due to the pH of the media affecting growth of the organism or actual enzyme activity. As the change in process pH was correlated to the level of ammonia in the fermentation broth it may be that the level of ammonia in the complex media affects lipase activity. The effects of soyaflour concentration, and therefore nitrogen level in the media, on lipase activity were investigated using pH controlled fermentations, Section 4.3. The lipase enzyme in *S. clavuligerus* has been reported to be associated with the cell membrane (Cavanagh, 1996). The rise in pH may alter the expression system of the enzyme, for example altering the cells outer membranes, or may affect the lipase enzyme directly.

Although lipase activity varied with changes in media pH, rates of rapeseed oil utilisation seemed relatively unaffected. Residual rapeseed oil levels remained the same in all fermentations at approximately 30-35% of initial oil concentrations. Effect of pH

on soyabean oil utilisation in the production of cephamycin C was investigated by Park *et al.* (1994). Residual soyabean oil was 20% of the initial oil concentration at the end of a fermentation held at pH 7.5. In contrast, when pH was controlled at 7.0 or 8.0 or without any pH control, the remaining oil concentrations were 67%, 38% and 42% respectively, of the initial oil available in the complex media. No noted effects of pH on growth were reported.

Antibiotic biosynthesis has also been reported to be affected by the type of pH control agents used in a process. Jensen *et al.* (1981) reported that when acetic acid was used in the feed of *Penicillium chrysogenum* to control pH penicillin (V) production increased 25% when compared to glucose only feed. Park *et al.* (1994) investigated the influence of several pH control agents on soyabean oil utilisation and antibiotic production. Variations in antibiotic titres were reported dependent on pH control agent used in fermentations of cephamycin C in soyabean oil media.

From data presented in this study and the known effects of changes in pH on growth and enzyme activity pH control is essential for reproducible fermentations of *S. clavuligerus* in relation to growth and therefore productivity. Selectivity of pH control agents is also critical to achieve optimum process control. Future fermentations were therefore pH controlled at a set point of 7.0 with acid (2M HCl) and base (30% ammonia solution).

#### **4.2. The effect of carbon source**

Addition of oil to fermentation broth has been shown to increase titres in many antibiotic processes, Section 2.4.3. The effects of part replacement of carbohydrate carbon sources with rapeseed oil on growth, lipase activity and clavulanic acid titres in a 5 L batch fermentation were investigated. A comparison was made between fermentations containing a carbohydrate only carbon source, modified starch, and a complex media containing both modified starch and oil as carbon sources. In the fermentation containing the mixed carbon sources a percentage of the modified starch was replaced by rapeseed oil on a carbon:carbon basis. The ratio of modified starch to oil used in each fermentation is shown in Table 4.1. It was noted that on a carbon:carbon basis rapeseed oil was ~~the~~ preferred in terms of volume as less oil was needed to provide the same amount of carbon than carbohydrate. Initial foaming problems during the 5 L batch fermentations minus rapeseed oil highlighted the use of oil, not only as an alternative carbon source but, a natural antifoam agent.

*S. clavuligerus* is unusual in its ability to utilise complex carbohydrate sources, such as starch, yet is unable to utilise simple sugars, such as glucose (Aharonowitz and Demain, 1978). Modified starch is a complex glucose polymer and is added to the fermentation media in conjunction with rapeseed oil to both increase initial cell mass and increase lipid dispersion in order for the rapeseed oil to be metabolised. The ratio of modified starch to rapeseed oil in the process media was investigated in order to study the effects on growth, lipase activity and rapeseed oil utilisation. Carbon levels were kept constant in all process media, Table 4.1.

**Table 4.1. Ratios of modified starch and rapeseed oil used in process media**

Modified starch	Rapeseed oil
7.9	0
2.0	2.0
1.0	2.3

No method is available for the assay of modified starch levels in the process media. As modified starch is a complex of glucose, glucose assays were carried out using a glucose hexokinase assay kit, (Sigma). Glucose detection using the Sigma supplied kit included acid hydrolysis and results on standard media preparations showed that carbohydrate was also present in the soya flour component of the complex media. Assays to measure the depletion of modified starch were not possible using this method. HPLC was also investigated as a method of modified starch utilisation. Detection was not possible again due to the carbohydrates in the soyaflour component of the complex media.

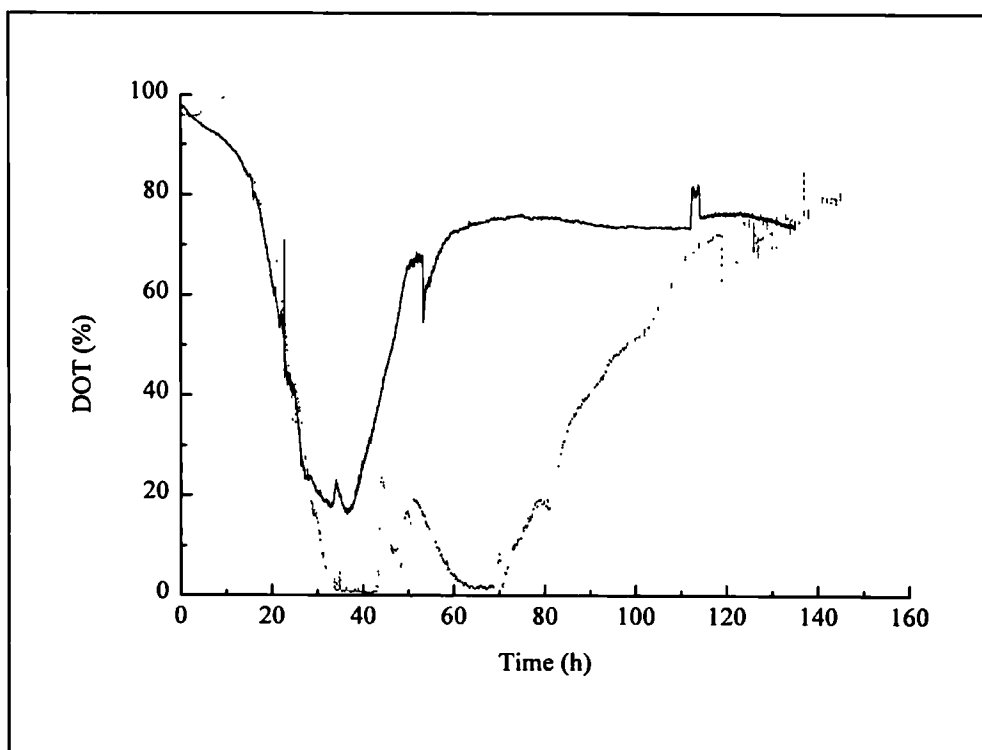
All carbon source comparison fermentations were carried out on a 5 L batch scale series 210 LH fermenter, Figure 3.1. The air flow rate was at a set point of 1 vvm and initial agitation at 625 rpm. pH was maintained at a set point of 7.0. Dissolved oxygen tension was maintained above approximately 10% by increasing agitation rates. On-line measurements included dissolved oxygen tension, carbon dioxide evolution rate and oxygen uptake rate of the fermentation. The respiratory quotient was then calculated.

#### **4.2.1. The effect of carbon source on growth**

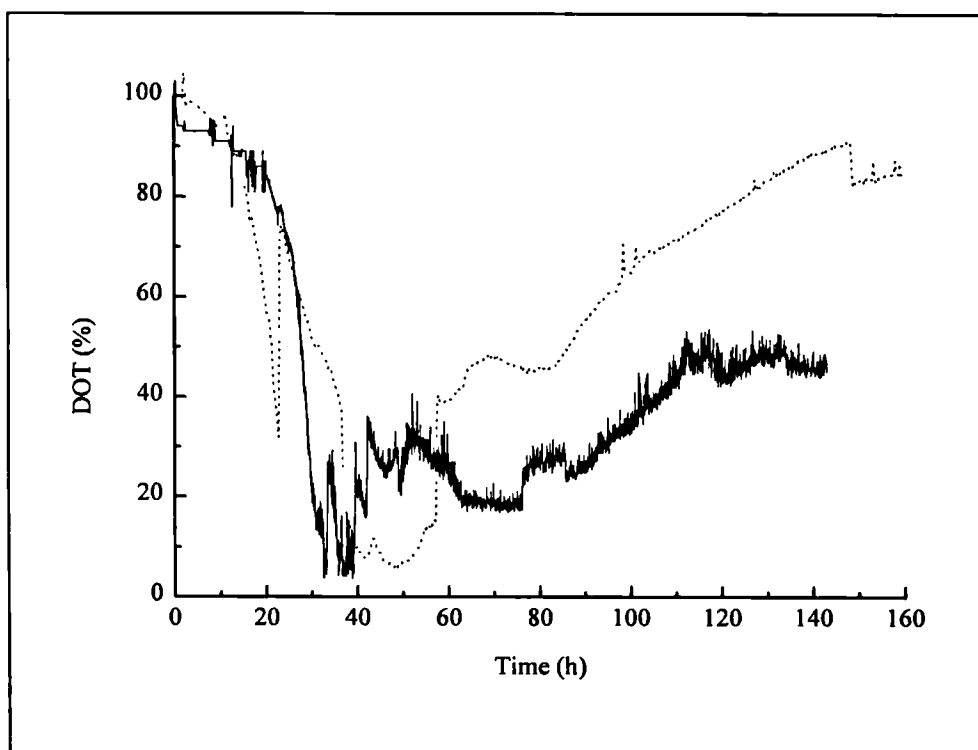
Due to the complex nature of the process media and the hyphal structure of *S. clavuligerus* growth traditional methods of biomass analysis were not possible. Changes in biomass were correlated to differences in DOT and exit gas analysis.

##### **4.2.1.1. Dissolved oxygen tension (DOT)**

Initial decline in dissolved oxygen in fermentations containing carbohydrate only carbon source and fermentations containing media with both oil and modified starch were very similar, Figure 4.6.a. Agitation rates were increased many times in both processes in order to maintain the DOT above 10%. This was successful in the fermentation containing a combination of carbon sources but not in the fermentation containing solely carbohydrate. The initial decline in DOT was too rapid and the agitation rate could not be increased at ~~such~~ <sup>SUFFICIENT TO MAINTAIN THE DOT ABOVE 10%.</sup> a rate due to the unknown affects of shear on the hyphal morphology of *S. clavuligerus*. It was concluded that *S. clavuligerus* utilises carbon for growth from a complex carbohydrate more rapidly than carbon in lipid form.



**Figure 4.6.a. Comparison of DOT measurements in fermentations containing a carbohydrate only (---) and combined carbohydrate and oil (-) carbon sources.**



**Figure 4.6.b. DOT profiles throughout batch fermentations containing 2.3:1.0 (-) and 2.0:2.0 (· ·) rapeseed oil:modified starch.**

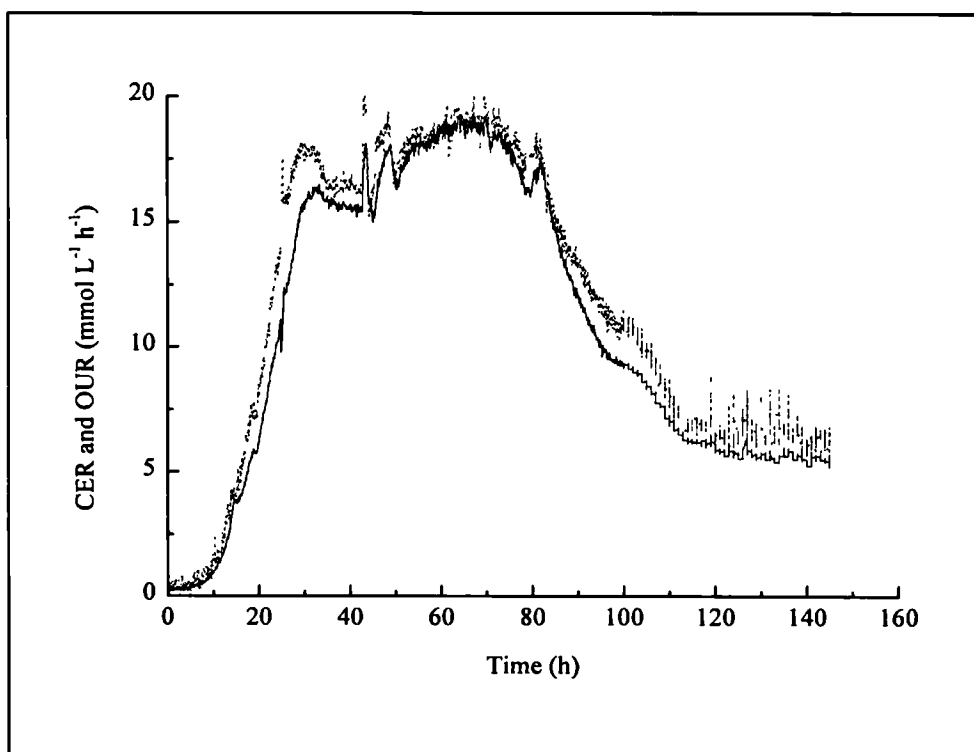
Figure 4.6.b. illustrates the trend in dissolved oxygen during fermentations containing a ratio 2.0:2.0 and 2.3:1.0 rapeseed oil:modified starch. The graph shows that the fermentation containing 2.0:2.0 had a more rapid initial decrease in DOT than in the fermentation containing 2.3:1.0. This was as expected due to the increase in the levels of complex carbohydrate available to the organism. The agitation rate in the 2.0:2.0 fermentation was increased, from 625 to 825 in 100 rpm stages, during the course of the fermentation, in order to maintain the DOT above a level of 10%. The agitation rate was increased at 32 h from 625 rpm to 725 rpm in the fermentation containing 2.3:1.0 rapeseed oil:modified starch. After 40 h the DOT in the fermentation containing 2.3:1.0 oil:starch begins to rise which may signify the end of growth on the carbohydrate only carbon source and a gradual shift towards oil metabolism.

From these data the ratio 2.3:1.0 is sufficient to increase initial biomass to a level which makes oil utilisation possible and will be used in future fermentations. 2 L shake flask experiments revealed that if the level of modified starch was decreased further or excluded from the complex media the increase in initial biomass was insufficient to sustain growth and metabolise the lipid component of the medium.

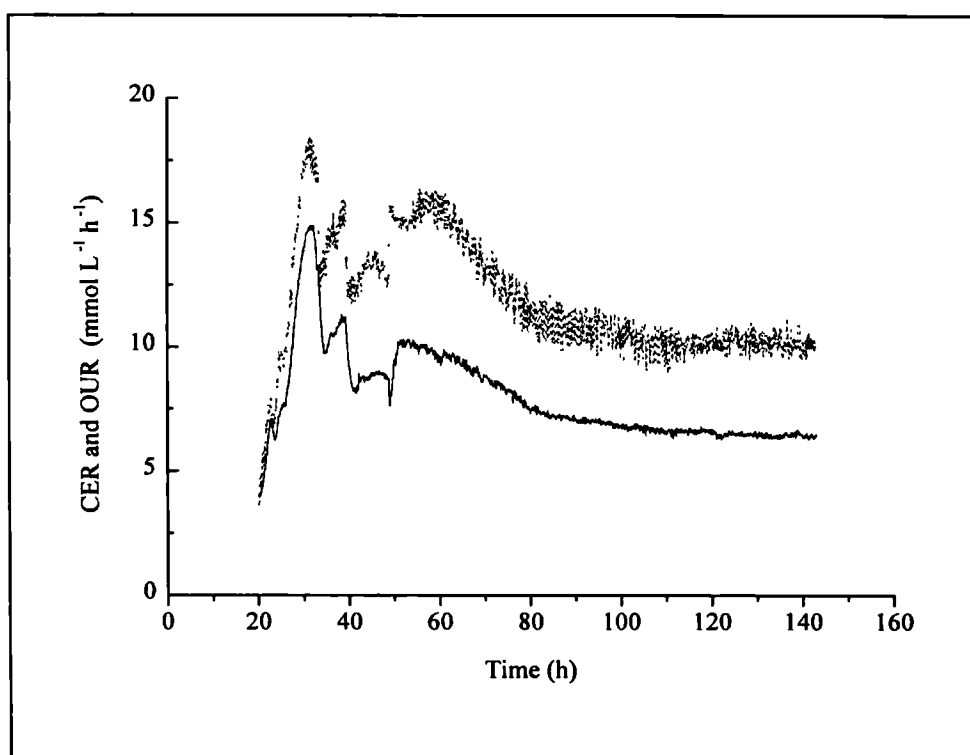
#### 4.2.1.2. Exit gas analysis

Exit gas analysis for fermentations containing carbohydrate only carbon source and the combined oil and carbohydrate are shown in Figure 4.7. On comparison the initial increase in OUR and CER were similar. The carbohydrate only carbon source has an extended stationary phase before the decline in exit gas values when compared to the process containing oil and carbohydrate in the media. This is due to the amount of rapidly catabolisable carbohydrate available. Rapid catabolism of a carbohydrate although responsible for extended growth, causes a decrease in the biosynthesis of many antibiotics (Park *et al.*, 1994). Oil is added to the media as a slow releasing carbon source due to the hydrophobic nature of the lipid molecule. The earlier decrease in OUR and CER in the combined oil/carbohydrate media indicates a switch in metabolism from the easily catabolisable modified starch to lipid. The switch decreases the rate of growth.





**Figure 4.7.a. Exit gas analysis for a fermentation containing only a carbohydrate carbon source. OUR ( ··· ) and CER ( - )**



**Figure 4.7.b. Exit gas analysis for a fermentation containing combined oil and carbohydrate carbon source. OUR ( ··· ) and CER ( - )**

Integration of the OUR profile gives a value for total oxygen used throughout each process. The oxygen requirement for utilisation of an oil substrate should be higher than that for a carbohydrate. In this study the carbohydrate only fermentation used a total of 1.5 mol oxygen and the complex oil/carbohydrate media 1.2 mol. Although more oxygen is required for oil metabolism it would be a more ~~comparative method~~<sup>appropriate</sup> if the comparison could be on a specific oxygen utilisation basis due to the differences in cell growth on both substrates. The calculation is based on integration under the curve and does not take into account incomplete oil utilisation. This was not possible due to the complex nature of the fermentation media. Respiratory quotient data is not shown due to OUR data noise. For the carbohydrate only fermentation the RQ value averaged approximately 0.8-0.9 throughout the process. For a complex oil/carbohydrate based media the RQ value should switch from approximately 0.9 to a value of approximately 0.6.

#### 4.2.2. Lipase activity

Lipase activity assays were carried out throughout the fermentation minus rapeseed oil. No lipase activity was detected. The presence of rapeseed oil in the process media either influenced expression of, or induced lipase activity.

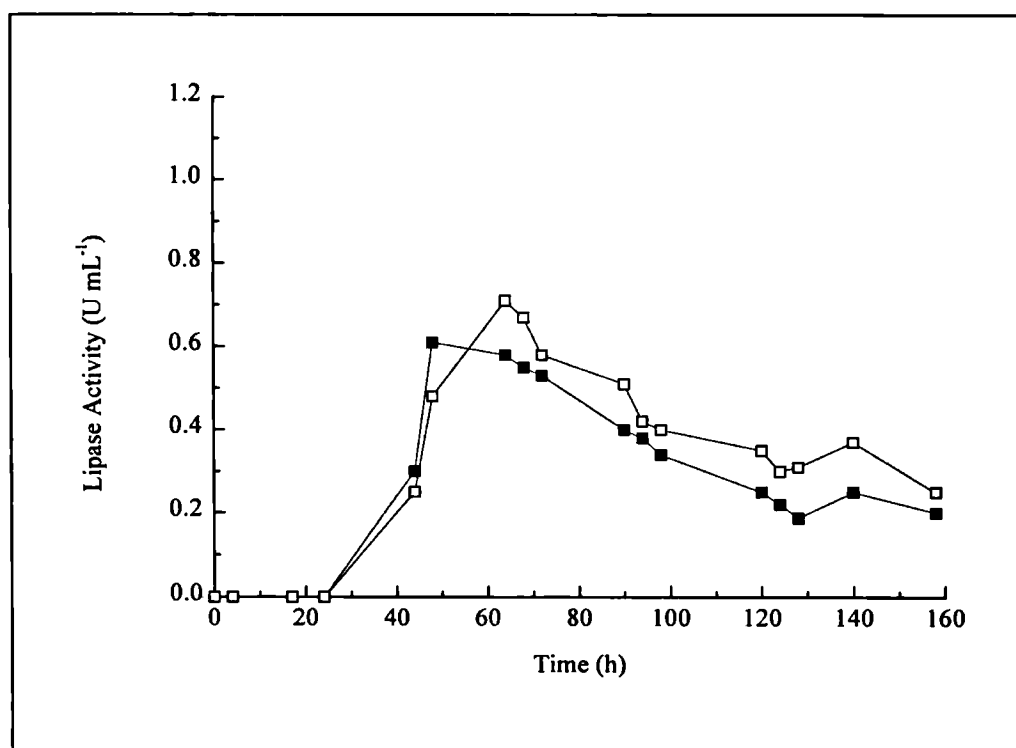


Figure 4.8. Lipase activity measured throughout fermentations containing 2.3:1.0(□) and 2.0:2.0 (■) rapeseed oil:modified starch.

Figure 4.8. shows that there was no significant difference in lipase activities measured in the fermentations containing 2.3:1.0 and 2.0:2.0 oil:modified starch. Lipase activity was detected after 24 h fermentation in both processes. Maximum lipase activity was slightly higher and appeared 20 hours later in the fermentation containing 2.3:1.0 oil:modified starch. Volumetric lipase activity was very similar between fermentations given the margin of error on a titrimetric assay.

#### 4.2.3. Total lipid analysis

Total lipid analysis revealed a more rapid initial utilisation of rapeseed oil in the process media when the level of modified starch was decrease from 2.0:2.0 to 2.3:1.0 oil:starch, Figure 4.9. A decrease in modified starch level would lead to earlier exhaustion in the carbohydrate carbon source and earlier lipid metabolism. Shake flask experiments revealed that if the level of modified starch is decreased further the carbohydrate level is too low to increase initial cell mass for lipid metabolism. Residual oil levels at the end of each fermentation were equal, approximately 30% of initial rapeseed oil.

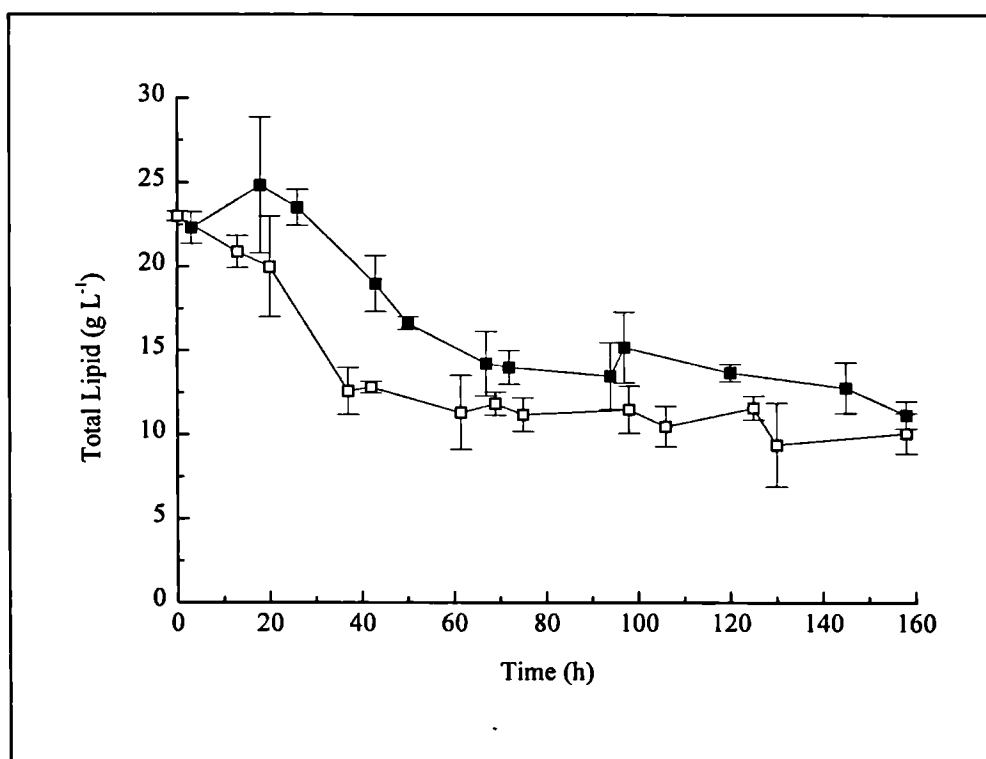


Figure 4.9. Total lipid analysis in 5 L batch fermentations containing 2.3:1.0 ( □ ) and 2.0:2.0 ( ■ ) rapeseed oil:modified starch.

The total lipid assay was carried out in the fermentations containing carbohydrate only carbon source to check the background level of lipid present. No lipids were detected in all samples assayed.

#### 4.2.4. Clavulanic acid titres

It has been reported that the addition of oil to a fermentation media as an alternative carbon source increases antibiotic production, Section 2.4.3. A comparison of clavulanic acid titres was made between process media containing a carbohydrate only carbon source and a fermentation under the same conditions containing a ratio of 2.3 rapeseed oil to 1.0 modified starch. Volumetric clavulanic acid titres are shown in Figure 4.10.a.

Figure 4.10.a. shows that clavulanic acid was produced after 40 h and was maintained at a level of approximately  $0.1 \text{ g L}^{-1}$  in the fermentation containing no rapeseed oil. A maximum peak was reached after 90 h of  $0.4 \text{ g L}^{-1}$ , although this single point may be due to assay error as problems were associated with HPLC column packing. When rapeseed oil was added to a 5 L *S. clavuligerus* batch fermentation clavulanic acid was produced after 20 h and increased at a much higher rate when compared to the fermentation containing no rapeseed oil. The final maximum titre was approximately  $1 \text{ g L}^{-1}$ . Variations in the fermentation conditions were minimised and therefore differences in titre can only be attributed to the presence of rapeseed oil in the complex media. Addition of rapeseed oil in combination with modified starch initiated clavulanic acid production earlier and increased the maximum clavulanic acid titre when compared to a fermentation containing a carbohydrate only carbon source.

Figure 4.10.b. shows the volumetric clavulanic acid titres in the fermentations containing 2.3:1.0 and 2.0:2.0 rapeseed oil:modified starch. The level of carbon in the media was constant, and the rapeseed oil to modified starch ratio varied, Table 4.1. Clavulanic acid titres showed that production was detected earlier and increased to a higher final titre in the process containing 2.3:1.0 than media containing 2.0:2.0 lipid:modified starch. Final clavulanic acid titre was approximately  $0.3 \text{ g L}^{-1}$  lower in the fermentation containing 2.0:2.0 oil:starch.

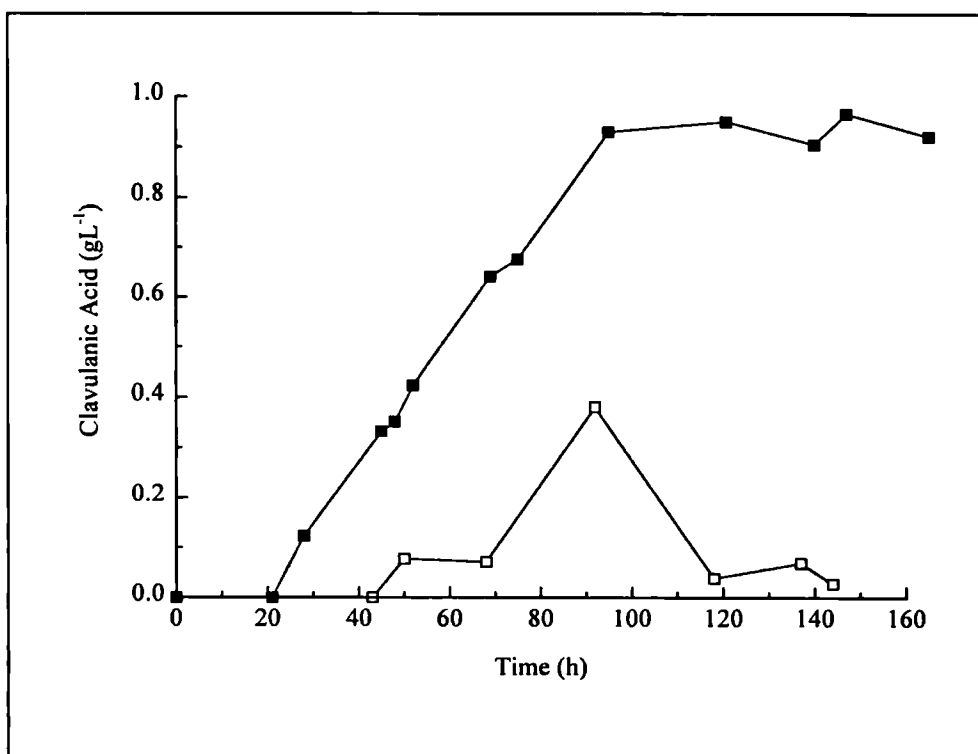


Figure 4.10.a. Comparison of clavulanic acid titres in fermentations containing a carbohydrate only carbon source (□) and combined carbohydrate and oil (■).

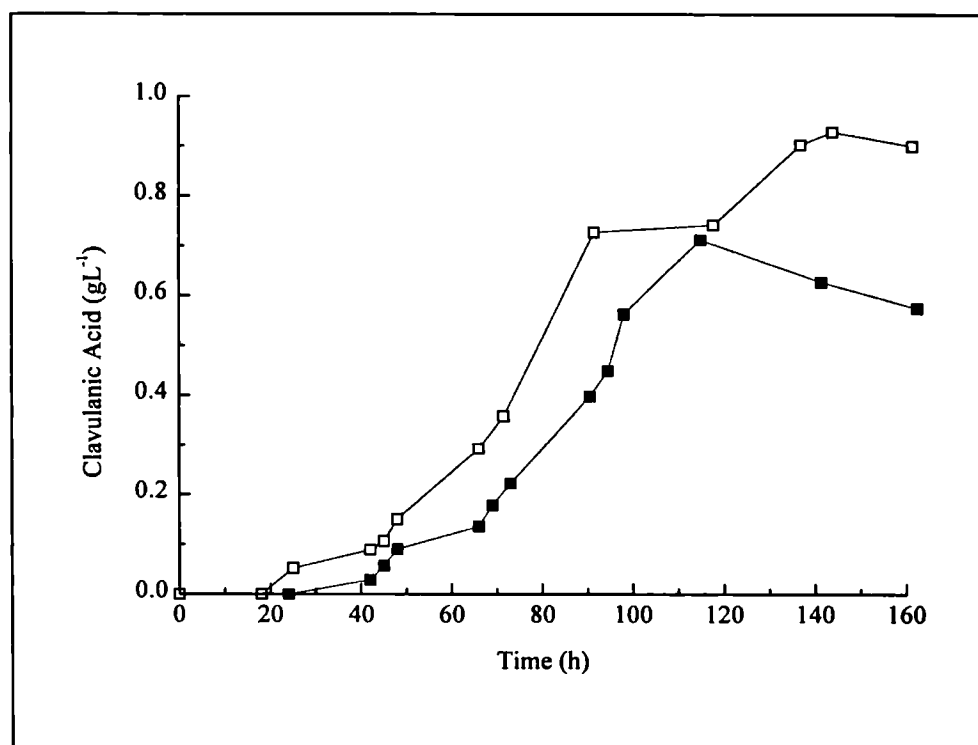


Figure 4.10.b. Clavulanic acid titres throughout fermentations containing 2.3:1.0(□) and 2.0:2.0 (■) lipid:modified starch.

Earlier production and increase in final product titre may be due to earlier metabolism of the rapeseed oil due to a decrease in modified starch level or an increase in the concentration of rapeseed oil present in the media. Decreases in final product titre may be attributed to changes in biomass. The decrease in final production of clavulanic acid may also be due to decomposition or remetabolism of the clavulanic acid. Simultaneous production and decomposition of clavulanic acid during *S. clavuligerus* fermentations in media containing glycerol and soy meal or soy meal extract has been reported by Mayer and Deckwer (1996).

A comparison of specific productivity would give a better comparison of production eliminating differences in growth patterns. Clavulanic acid titres seem unaffected by very low dissolved oxygen tensions seen in the comparison of DOT in Figure 4.6.b.

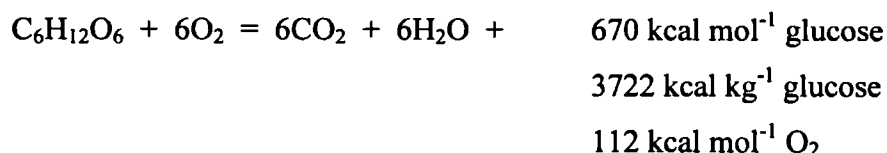
#### 4.2.5. Discussion

Carbon catabolites have been reported to repress penicillin biosynthesis in *Penicillium chrysogenum* (Revilla *et al.*, 1984) and cephalosporin production (Ahanowitz and Demain, 1978). Glucose is the usual carbon and energy source used in fermentation for growth of many antibiotic producing organisms. Rapid catabolism of the glucose causes a decrease in antibiotic production known as the 'glucose effect'. Catabolite repression is reduced in oil supplemented media as the oil concentration remains low in the process due to its hydrophobic nature. Data presented in this study showed the ease of metabolism and therefore growth, in a modified starch based media, Figure 4.7.a, and the effect of rapeseed oil addition, Figure 4.7.b. Initially cell growth was equal due to initial carbohydrate metabolism. The switch to oil metabolism is indicated by a decrease in OUR, Figure 4.7.b. Although modified starch, used as a sole carbon source, sustains rapid growth, oil addition represses rapid growth which may be the cause of increased antibiotic production.

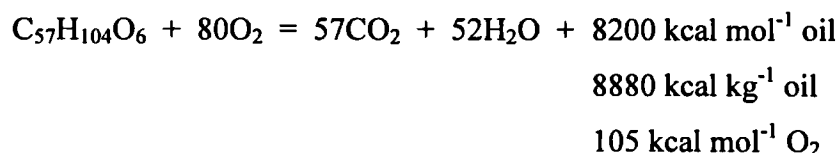
From the pathway of metabolism of clavulanic acid shown in Figure 2.2. the advantages of a lipid source can be seen. Products from  $\beta$ -oxidation include glycerol, acetyl CoA and, if an odd numbered carbon chain enters the  $\beta$ -oxidation cycle, propionyl CoA. All of these compounds have been shown to be necessary in clavulanic acid production. Although these may be supplied to the clavulanic acid biosynthesis pathway via carbohydrate metabolism the amount may be greatly increased through lipid catabolism.

Integration of the OUR profile gives an indication of total oxygen consumption during the fermentations. Energetically a typical oil requires 2.4 times as much energy on a volume basis and is less well oxidised than a carbohydrate (Stowell, 1987). Hence for a given energy input the oil has a higher oxygen demand.

Modified starch oxidation can be described as:



whereas oxidation of a typical C18:1 based triacylglyceride can be described as:



This is a very real consideration for many antibiotic processes using a lipid based production media where oxygen can be the limiting nutrient.

In this study oxygen consumption was lower in the fermentation containing oil than the carbohydrate only media. This is due to the differences in growth rates and ease of metabolism on each substrate. Lee and Ho (1996) investigated the effects of different lipids on growth and clavulanic acid production in *S. clavuligerus*. Strains tested grew well on palm oil and its fractions and glycerol as a main carbon source. A sucrose control produced poor growth and no clavulanic acid. The amount of growth on various carbon sources was found to be inversely proportional to clavulanic acid production. This supports the data presented in this study as growth on the carbohydrate based media was increased, when compared to an oil and carbohydrate carbon source, and clavulanic acid production was increased, Figure 4.10.a.

Change in the level of carbohydrate in the fermentation media delayed utilisation of the lipid due to the ease of modified starch metabolism. There was little change in detected lipase activity. However, clavulanic acid was produced earlier and with increased final

titre in the fermentation containing less modified starch. Earlier production and increased titres, may be due to earlier oil metabolism. A rapidly catabolisable carbon source such as modified starch in the case of *S. clavuligerus* helps to build up biomass rather than increasing antibiotic production.

The availability of insoluble substrates is limited by the degree of dispersion in the aqueous phase. Tan and Gill (1987) observed that, during growth of *Pseudomonas putida* on animal fats, initial growth rates were slow due to coalescence of the fat droplets. Addition of modified starch to the media reduced the lag phase otherwise observed due to initial coalescence of the rapeseed oil droplets. The reason for preferential use of the modified starch before the oil may be linked to the dispersion of the oil in the media. Initially, the dispersion of the oil is irregular as shown by the total lipid assay data error bars. As the fermentation continues the oil becomes more dispersed in the media and may therefore be more accessible to the cell.

In order to investigate the effect of process variables on lipid utilisation it is important to understand lipase activity. In some organisms lipase activity is induced by the presence of lipids but in others lipids may not be required for expression of activity or may inhibit it. Results from this study suggest that the lipase activity in *S. clavuligerus* is induced by the presence of rapeseed oil as no activity was detected in fermentations containing carbohydrate only carbon source.

Lipase biosynthesis has been detected without the presence of lipids in *Aspergillus niger* (Ohnishi *et al.*, 1994). When oils were added to the media the level of activity increased 3 fold when compared to the lipid free case. Lipase activity in a lipid free media has also been noted in 5 L batch fermentations of *Saccharopolyspora erythraea* (Mirjalili *et al.*, 1998) with further addition of rapeseed oil increasing detected lipase activity. Other reports suggest *de novo* synthesis of lipase in *A. niger* in the presence of lipid which was completely repressed by the addition of glycerol and glucose. It was concluded that it was the carbon chain moiety of the fatty acid present in the triacylglyceride that controlled lipase synthesis. Similar results have been observed in *Fusarium oxysporum* (Rapp, 1995). Lipase activity in Pseudomonads have also been reported to be inducible (Tan and Gill, 1985). Their induction does not require, and may be inhibited by the presence of fat in the media.



Lipase activity is organism dependent. It may be induced by the presence of lipids, produced constitutively or repressed by lipids in the process media. There may be one or more lipase enzyme present which may act independently, one being induced by the presence of lipid the other produced constitutively. Lipase activity in *S. clavuligerus* is only detected when required, that is in the utilisation of oil in the absence of a more readily available carbohydrate carbon source. Detected levels of activity seem relatively independent of rates of rapeseed oil utilisation in 5L batch fermentations of *S. clavuligerus*.

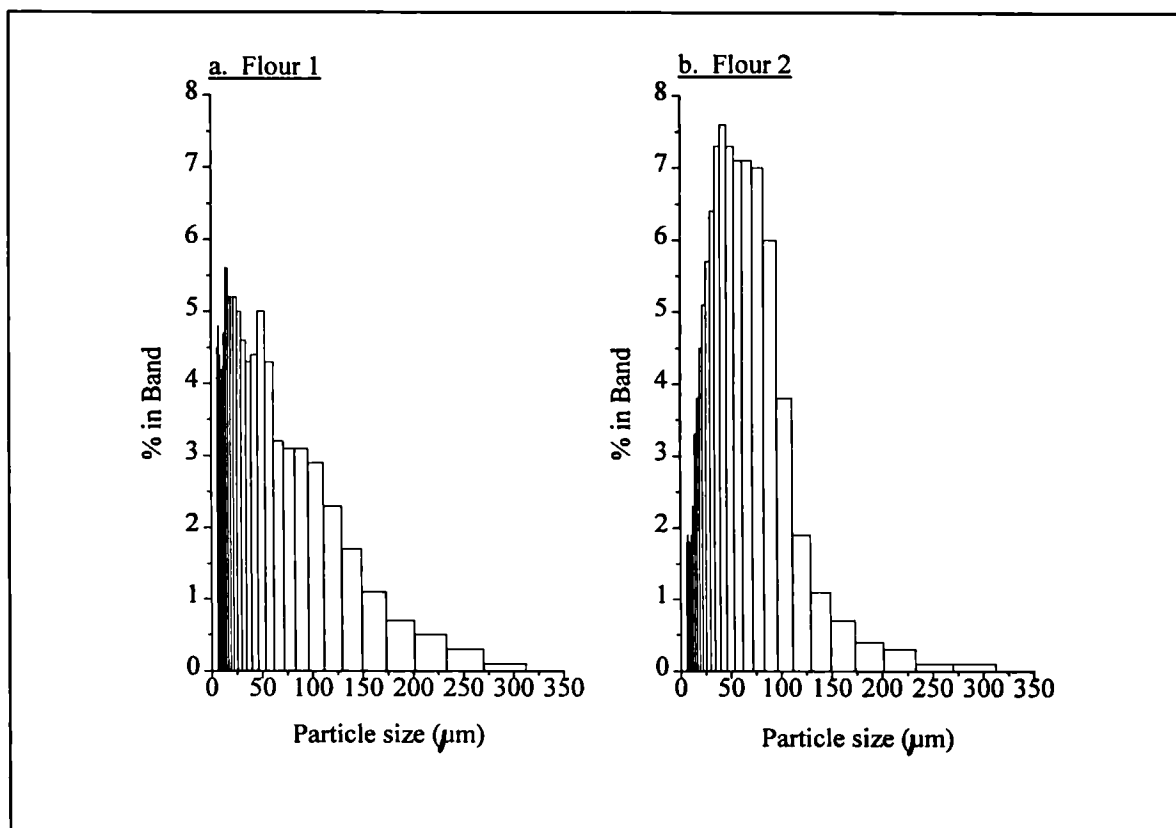
Data presented in this study suggest that rapeseed oil is utilised by induced lipase activity and lipid addition as a carbon co-substrate increases clavulanic acid production in 5 L batch fermentations of *S. clavuligerus*.

### **4.3. The effect of soya flour concentration and soya flour type**

Fermentations were carried out to investigate the effect of variations in the concentration of soyaflour and flour type. Two types of flour were compared flour 1 and flour 2. Flour 2 was a refined version of flour 1 both in terms of particle size, Figure 4.11., and nutrient content. Flour 2 contains approximately 70% protein whereas flour 1 contains 50%. Concentrations of flour are indicated by a relative ratio. For example 3:1 indicates three parts flour to one part starch. Fermentations were carried out with the concentrations of flour ranging from 1:1 to 3:1 and were analysed. rapeseed oil concentration was kept the same. Data regarding production of clavulanic acid was not obtained due to development of the chromatography assay.

#### **4.3.1. Particle size analysis**

Particle size distribution of flour 1 and flour 2 were analysed. Particle sizes ranged from 5  $\mu\text{m}$  to 350  $\mu\text{m}$ . The analyser used predetermines the size band and the results were plotted in histogram form, Figure 4.11. The area of each column represents the percentage of particles distributed within a defined size range.



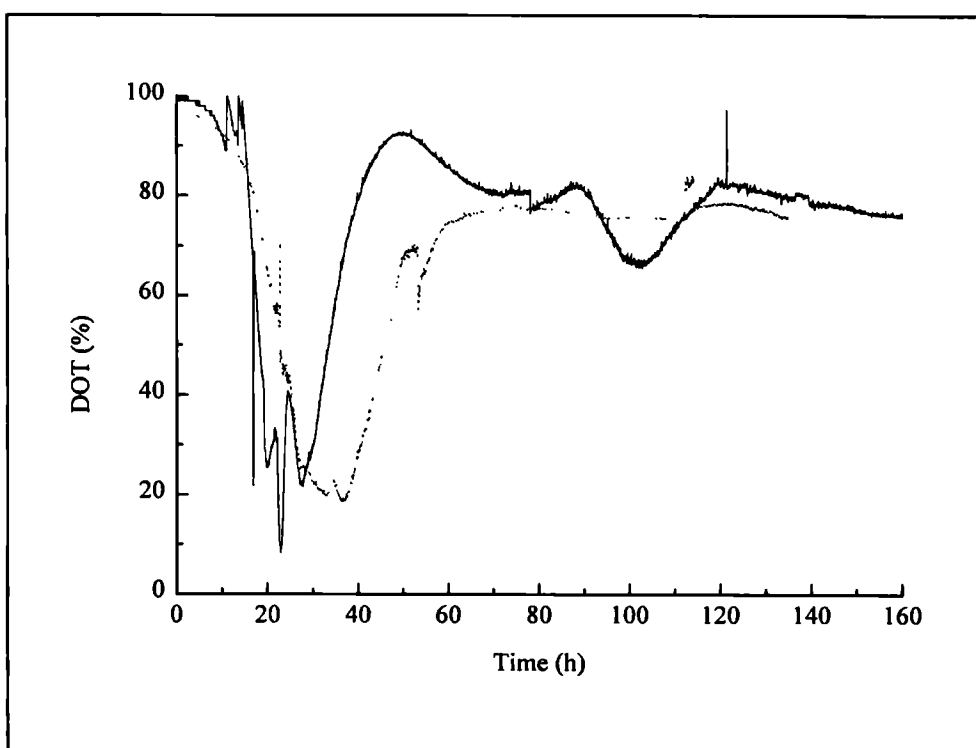
**Figure 4.11. Particle size distribution of flour 1 and flour 2 measured using laser diffraction technique.**

Particle size distribution within each flour was calculated on a percentage volume basis. Data illustrated that flour 1 had a wider range of particle sizes. The maximum percentage of particles in one size band in flour 1 was approximately 5.5 %. Flour 2, when compared to flour 1, had a larger percentage of particles within a narrower range of particle sizes. Flour 2 had a fewer percentage of particles within the smallest, less than 25  $\mu\text{m}$ , and largest, greater than 200  $\mu\text{m}$ , particle size ranges. The maximum percentage of particles with in a defined size band for flour 2 was approximately 7.5%. The narrower spectrum of particle sizes was as expected as flour 2 is a refinement of flour 1 in terms of particle size and nutrient content.

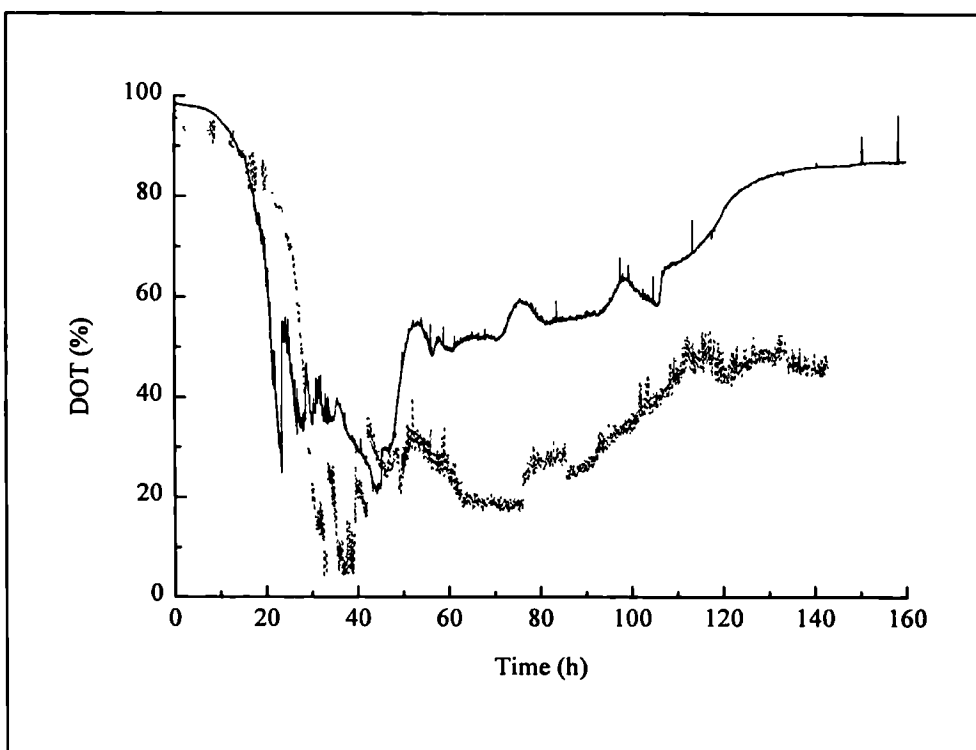
#### **4.3.2. The effect of flour type and concentration on growth**

All flour comparison fermentations were pH controlled with both acid and base in a 7 L LH 210 series vessel, Figure 3.1. Complex process media was used varying the concentration and type of soyaflour as noted. Differences in growth between fermentations was measured using dissolved oxygen tension. Exit gas analysis was incomplete due to equipment and data acquisition failure. DOT was maintained above 10% by increasing agitation rates from 625 to 925 rpm in 100 rpm steps where necessary.

Initial decline in DOT was similar for the fermentations containing different concentrations of flour 1, Figure 4.12.a. Carbon content, in terms of modified starch and oil, was identical. The decline in DOT from 20-30 h was more rapid in the fermentation containing 3:1 than 2:1 flour. Flour 1 contains 32% carbohydrates and the more rapid decline in DOT may be due to the increase in carbohydrate present ~~with the~~ in flour. The same conclusion can be drawn from the DOT profiles from the fermentations containing different concentrations of flour 2, Figure 4.12.b. DOT increases after approximately 40 h and remained at a level of 80% in the fermentations containing flour 1 and 3:1 flour 2. The 2:1 flour 2 process has a final DOT of approximately 50%. This may be due to the fact that the agitation rate was not increased at the same rate in this fermentation which will affect oxygen transfer rates.



**Figure 4.12.a. DOT profile for fermentations containing 2:1 (.....) and 3:1 (—) flour 1.**



**Figure 4.12.b. DOT profile for fermentations containing 2:1 (.....) and 3:1 (—) flour 2.**

At a level of 1:1 the flour was at an insufficient level to sustain growth. Figure 3.1.c. Exit gas analysis was not complete for any flour comparison fermentations due to equipment and data logging failure.

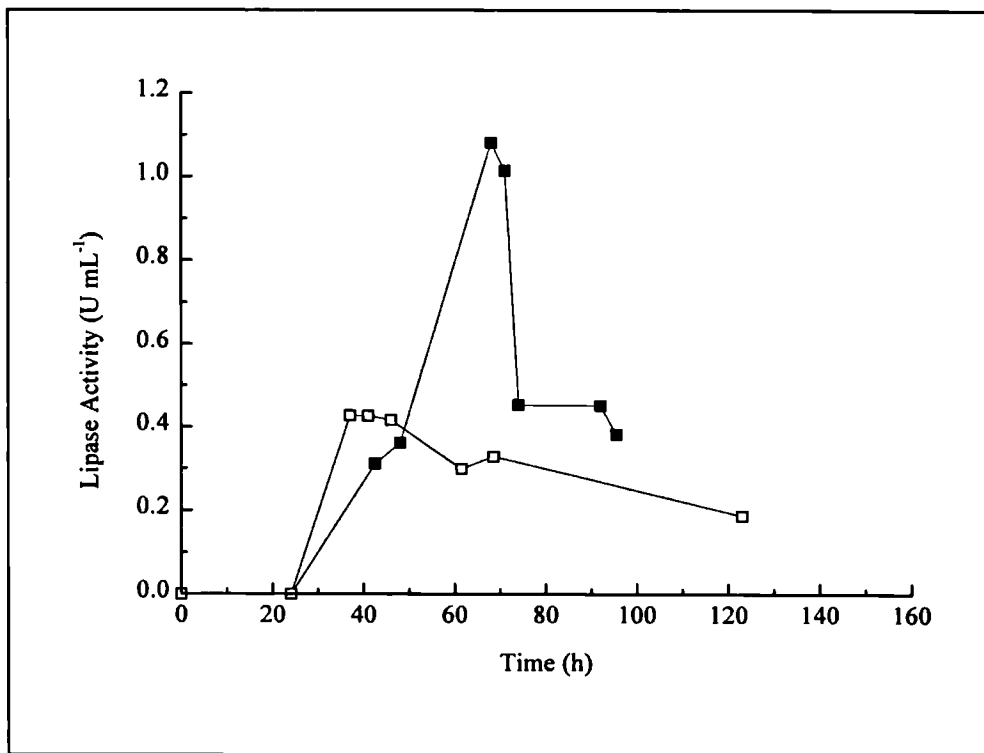
#### 4.3.3. Lipase activity

The effect of soya flour concentration and type on lipase activity was investigated, Figure 4.13. Data showed that as the level of flour 1 in the media was decreased the level of detected lipase activity also decreased, Figure 4.13.a. Maximum lipase activity in the fermentation containing 2:1 flour 1 ( $0.42 \text{ U mL}^{-1}$ ) was substantially less than in the media containing 3:1 ( $1.05 \text{ U mL}^{-1}$ ). No activity was seen in the fermentation containing 1:1 as this level of soyaflour was insufficient to sustain growth after 30 h.

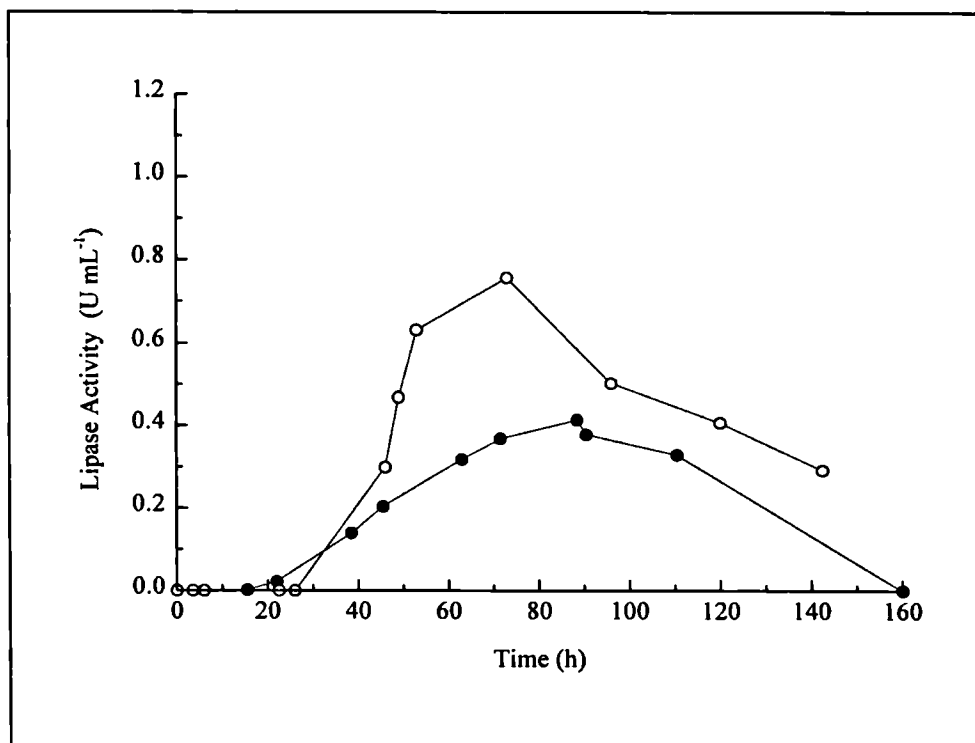
Lipase activity within a fermentation containing 3:1 flour 2 was substantially lower when compared to flour 1, Figure 4.13.b. When the concentration of flour 2 was reduced to 2:1 the level of lipase activity increased. This difference may be due to the refinement of the flour 2. In terms of protein, 3:1 flour 1 is equivalent to 2.14:1 flour 2 as flour 2 contains approximately 70% protein and flour 1 only 50%. Lipase activity for the fermentation containing 2:1 flour 2 should show similar levels to the fermentation containing 3:1 flour 1.

An increase in the concentration of flour 2, from 2:1 to 3:1, decreased detected lipase activity. The increased protein content of the media, due to the refinement of flour 2, may be increasing the protein to a level which may inhibit lipase activity. An increase in protein content may increase the detected levels of ammonia present in the fermentation broth which may also affected detected lipase activity.

Control titrimetric experiments could be carried out using different concentrations and type of soyaflour spiked with the same amount of standard lipase. Lipase activity data would show if activity was affected by soyaflour concentration and type. A standard lipase preparation from *S. clavuligerus* was not obtained as it is reported not to be secreted into the surrounding media (Cavanagh, 1996).



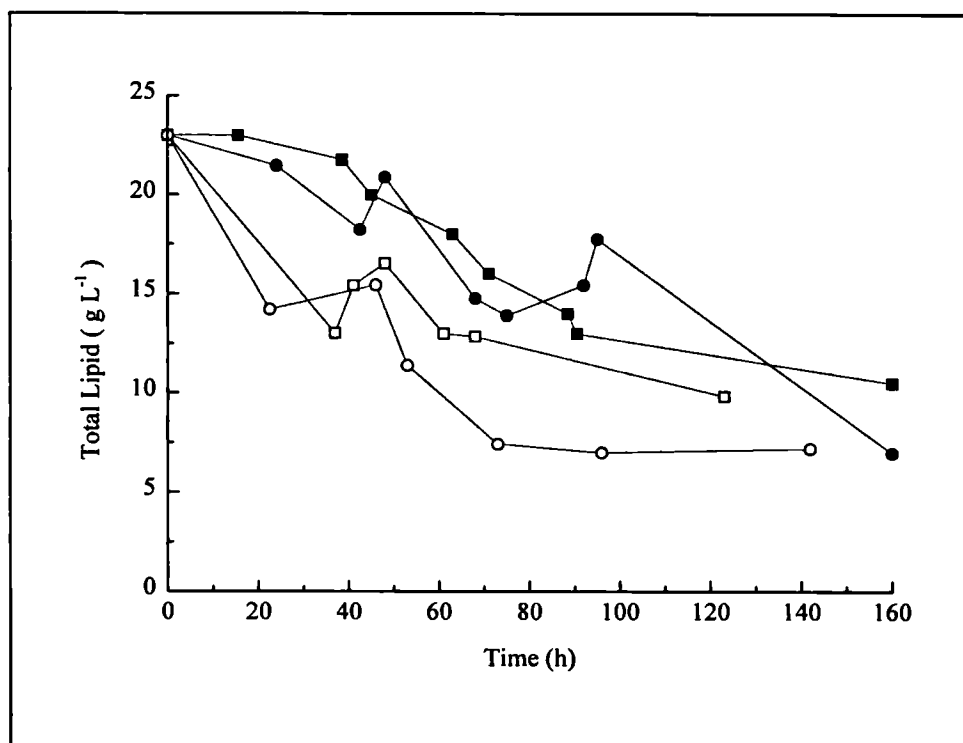
**Figure 4.13.a. Lipase activity in fermentations containing 2:1 (□) and 3:1 (■) flour 1**



**Figure 4.13.b. Lipase activity in fermentations containing 2:1 (○) and 3:1 (●) flour 2**

#### 4.3.4. Total lipid analysis

A wide variability in lipase activity was seen when comparing concentrations and type of soyaflour used in the complex media. Utilisation of the rapeseed oil content of the media and residual oil levels at the end of fermentation were analysed, Figure 4.14. Data showed that although lipase activity varied considerably residual rapeseed oil levels in fermentations containing different concentrations and types of soyaflour were relatively unaffected.



**Figure 4.14. Total lipid utilisation in fermentations containing 2:1 flour 1 (□) and flour 2 (○) and 3:1 flour 1 (■) and flour 2 (●).**

Data showed that initial utilisation rates of rapeseed oil were more rapid when the concentration of flour used was 2:1 when compared to 3:1. A decrease in flour concentration increased initial oil utilisation rates. This may be due to the way in which the particles of flour are distributed in the fermentation and interact with the oil droplets increasing dispersion and hence utilisation rates. An increase in flour concentration may inhibit utilisation by coating the oil droplet, decreasing the interface available which is essential for lipase activity.

#### 4.3.5. Discussion

As no method for comparing specific lipase activity was available and noted changes in DOT were similar, lipase activities were compared on a volumetric basis. The increase in the level of flour 1 within the media, from 2:1 to 3:1, increased the level of lipase activity. An increase in the concentration of flour 2 in the media, from 2:1 to 3:1, decreased detected lipase activity. This may be an example of metabolic inhibition of lipase activity by increased protein content. The effect of levels of nitrogen in media on lipase activity have been investigated in *A. oryzae* (Ohnishi *et al.*, 1994). An optimum concentration of polypepton was found, above and below which reduced lipase activity was detected. Salts, amino acids, proteins and urea were used as nitrogen sources in media used to determine their effect on lipase activity in *Rhizopus oligosporus*. Lipase activity was not stimulated except in the presence of soyabean meal extract. Peptides have also been shown to enhance lipase activity in *Staphylococcus aureus* (Mates and Sudalievitz, 1973).

A study on the effect of nitrogen sources in batch cultivation to produce lipase from *Candida rugosa* (Montesinos *et al.*, 1996) observed that if nitrogen assimilation by the cells was reduced the lack of nitrogen produced a decrease in protein synthesis. A decrease in protein synthesis meaning lipase synthesis was also suppressed. In batch cultures there was no observed effect of change in nitrogen source on lipase production. Changing nitrogen source in continuous culture increased nitrogen assimilation, which in turn increased protein production and hence increase lipase productivity.

Type of soyaflour used, although affecting lipase activity, had little effect on lipid utilisation. A decrease in concentration of flour increased initial oil utilisation rates. Distribution of flour particles within the media may play a role in the rate of lipid utilisation due to interactions between the oil droplets and the flour particles. When the concentration of flour was decreased to 2:1 from 3:1 initial lipid utilisation was more rapid. This may be due to the way in which the flour particles are distributed and interact with the droplets of oil. At the lower concentration the flour may form a thin coating around the oil globules in a way which prevents oil droplets coalescing and therefore making the oil interface more accessible to the lipase enzyme. If the concentration of flour is increased the flour may produce a thicker coat around the oil droplet covering the interface and preventing lipid utilisation. Alternatively the oil may



coat the flour particles and the fewer particles there are the larger the oil droplet and the larger the initial interface. If the oil coated the flour particles the increase in flour concentration would be expected to increase lipid utilisation as the lipid droplets surface area to volume ratio would increase.

Clavulanic acid titres were not measured in these experiments due to the development of the HPLC method. The level of media components, such as nitrogen, may have an effect on the level of antibiotic produced. When ammonium or phosphate ions were provided to *S. clavuligerus* at levels satisfying maximum growth secondary metabolism was suppressed (Fang and Demain, 1995). The interference in production occurs under otherwise optimum conditions for antibiotic production including high levels of aeration. The study concludes that ammonium and phosphate ions do not interfere with  $\beta$ -lactam production and may actually stimulate secondary metabolism under low levels of aeration. The mechanisms of regulation are as yet unclear.

Although the rates of lipid utilisation are different between flour concentrations the residual oil level is unaffected by either changes in the concentration or type of soya flour used. This suggests that physical variables, and not media components, may be the key to reducing residual oil levels.

**4.4. The effect of lipid source on growth and lipase activity**

Shake flask experiments were carried out in order to investigate the effect of the individual triacylglycerides present in rapeseed oil, and their respective fatty acid methyl esters, on growth and lipase activity in *S. clavuligerus*. Rapeseed oil contains 5 fatty acids, Table 4.2., which vary in chain length and level of saturation. This investigation was carried out in order to determine differences in utilisation between chain length and saturation level. Triacylglycerides investigated were triolein, tristearin and tripalmitin. Fatty acid methyl esters of triolein and tristearin were also investigated with respect to growth and lipase activity.

**Table 4.2. Composition of rapeseed oil.** (Data supplied by SmithKline Beecham)

Fatty acid	Structure	% of rapeseed oil
Palmitic	C16:0	5
Stearic	C18:0	1.5
Oleic	C18:1	61
Linoleic	C18:2	22.5
Linolenic	C18:3	10

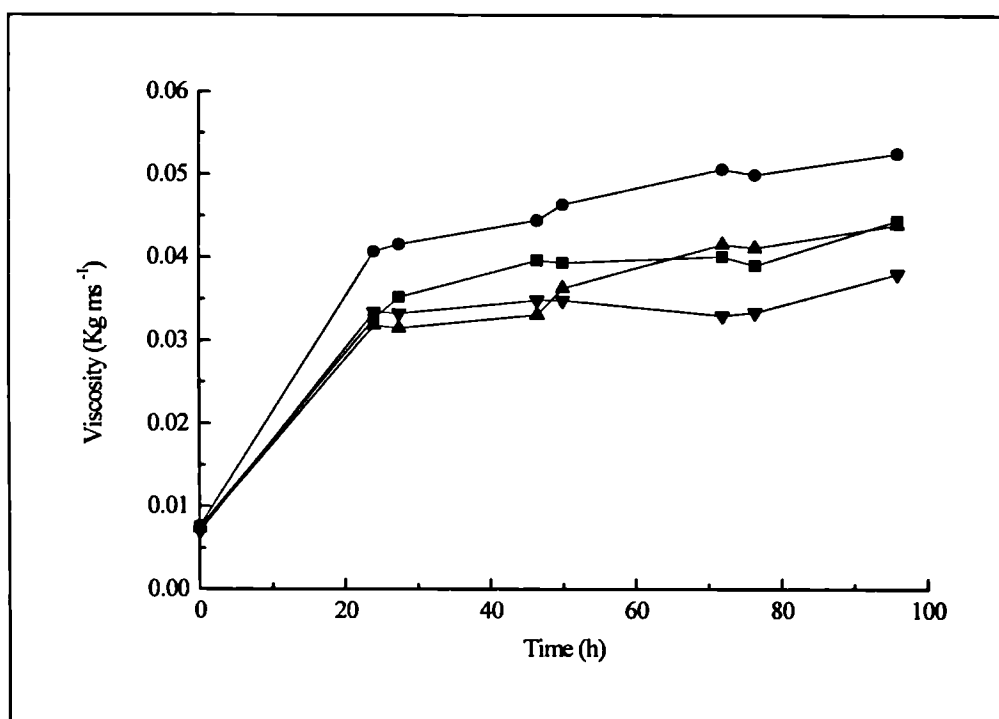
2 L shake flasks contained 200 mL of complex media, Section 3.1.9., containing 1.5:3.4 triacylglyceride or methyl ester:modified starch. This was equivalent to the carbon ratio used on 5 L pilot plant scale studies. Palmitic acid was replaced on a weight:weight and not carbon:carbon basis as the difference in carbon level was thought insignificant. Changes in biomass and lipase activity were reported. Total lipid analysis was not carried out on this scale as sampling would reduce total volume in the shake flask to an unacceptable level. Clavulanic acid assays were carried out but no clavulanic acid was detected on such a small scale. Rapeseed oil was used as a control in all shake flask experiments. Experiments were carried out in duplicate and average results presented.

## Summary of shake flask experiments :

1. Rapeseed oil (control), triolein, tristearin, tripalmitin.
2. Rapeseed oil (control), methyl stearate, tristearin.
3. Rapeseed oil (control), methyl oleate, triolein.

**4.4.1. The effect of lipid source on growth**

Changes in biomass levels in the shake flask experiments were reported in terms of changes in broth viscosity, Figure 4.15. Viscosity has previously been shown to be a valid method used to measure specific enzyme activities in Streptomyces fermentations (Sarraf *et al.*, 1996). On comparison of the individual triacylglycerides, Figure 4.15.a., triolein was the better carbon source for growth of *S. clavuligerus*, than rapeseed oil, tristearin and tripalmitin. Differences in biomass levels may also be due to the physical state of the triacylglyceride. At incubation temperature, 26°C, triolein was liquid, tristearin a fine powder and tripalmitin was flakes ranging up to approximately 3mm<sup>2</sup>. When compared to the rapeseed oil control, which was also liquid at 26°C, tristearin had similar biomass levels and therefore physical state did not affect growth or viscosity measurements. Triolein enhanced biomass levels when compared to the rapeseed oil control. Tripalmitin was added on a weight:weight basis not carbon:carbon but the difference was not large enough to warrant the noted effect on final biomass levels.



**Figure 4.15.a. Viscosity measurements for shake flask experiments containing rapeseed oil (■), triolein (●), tristearin (▲), tripalmitin (▼).**

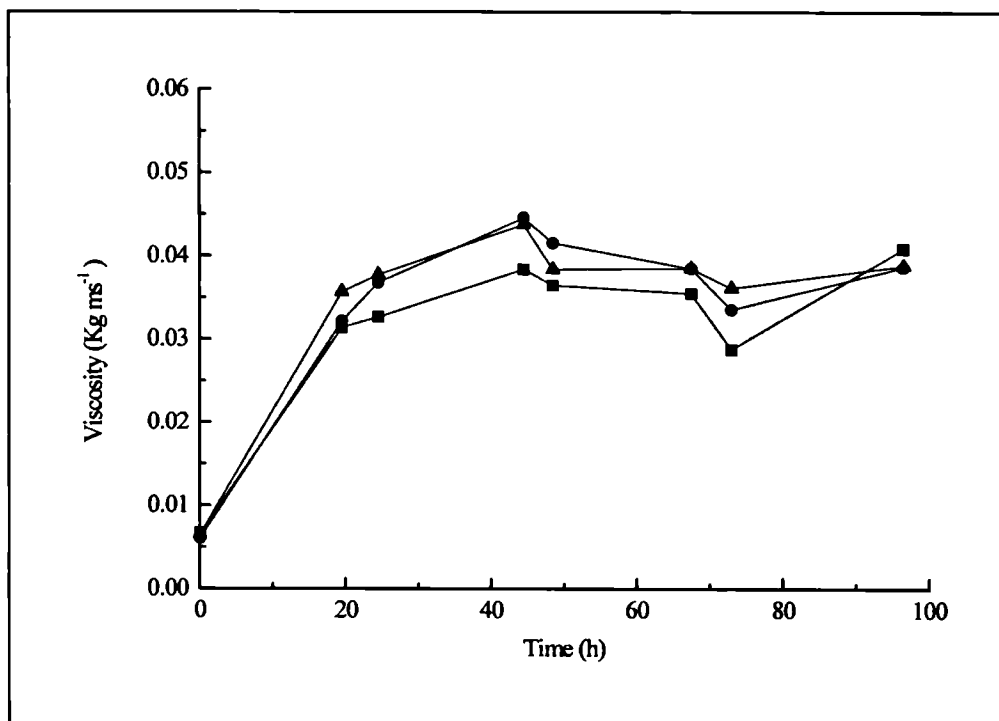


Figure 4.15.b. Viscosity measurements for shake flask experiments containing rapeseed oil (■), tristearin (●), methyl stearate (▲).

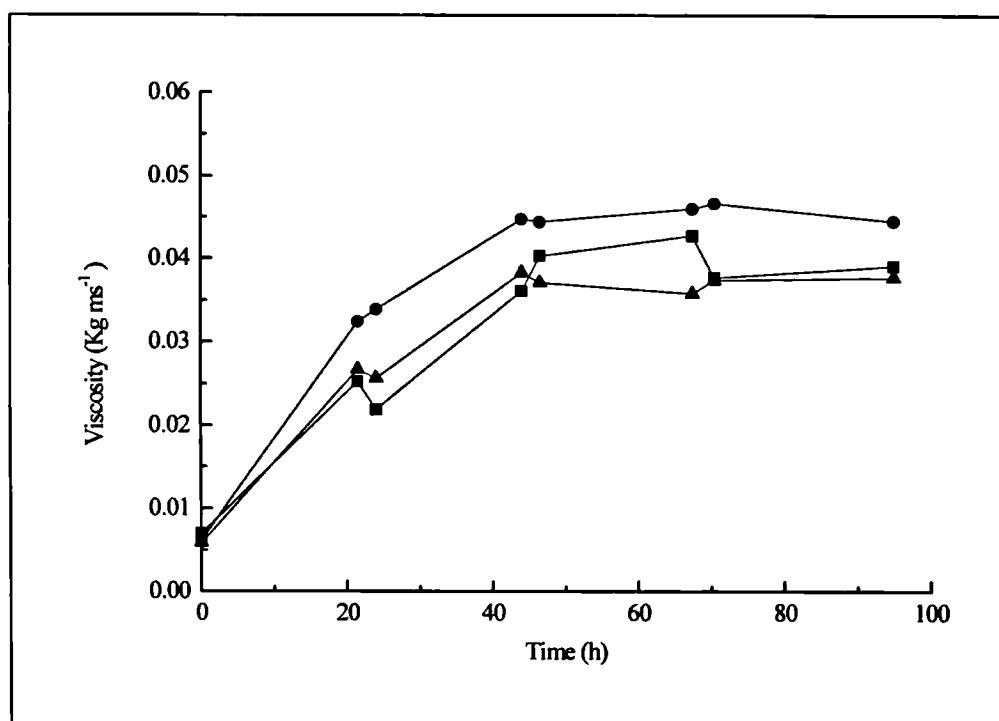


Figure 4.15.c. Viscosity measurements for shake flask experiments containing rapeseed oil (■), triolein (●), methyl oleate (▲).

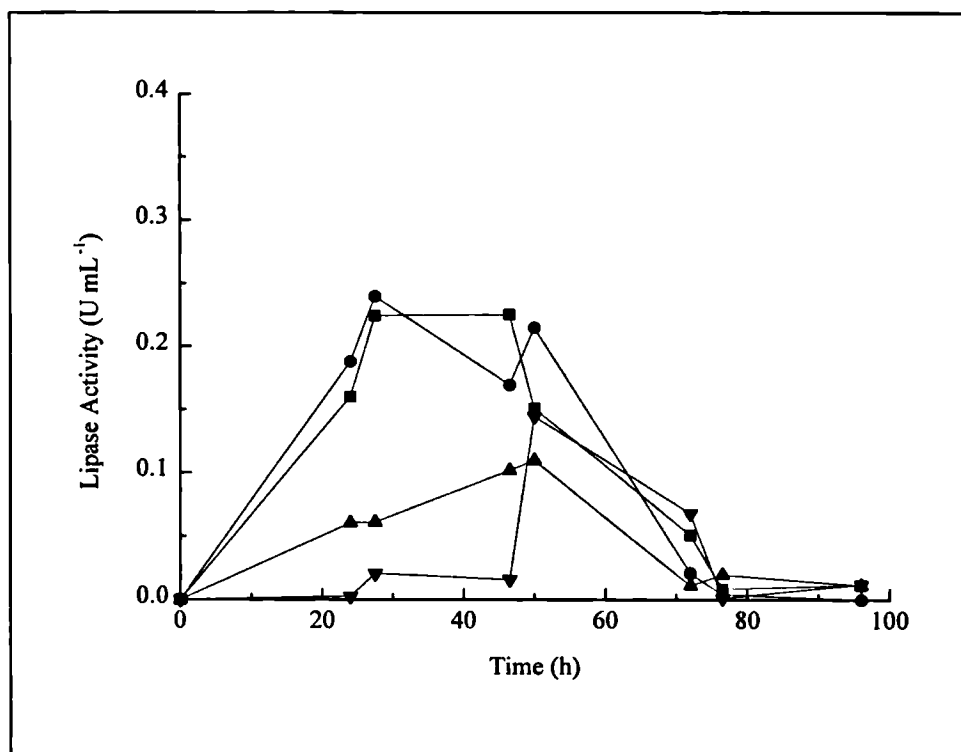
Methyl stearate and tristearin supported similar growth when compared to a rapeseed oil control, Figure 4.15.b. Final biomass levels were approximately equal. Triolein enhanced biomass levels when compared to methyl oleate and a rapeseed oil control, Figure 4.15.c. Fatty acid methyl esters were added as fine powders but the physical state did not effect the achieved biomass levels.

#### 4.4.2. The effect of lipid source on lipase activity

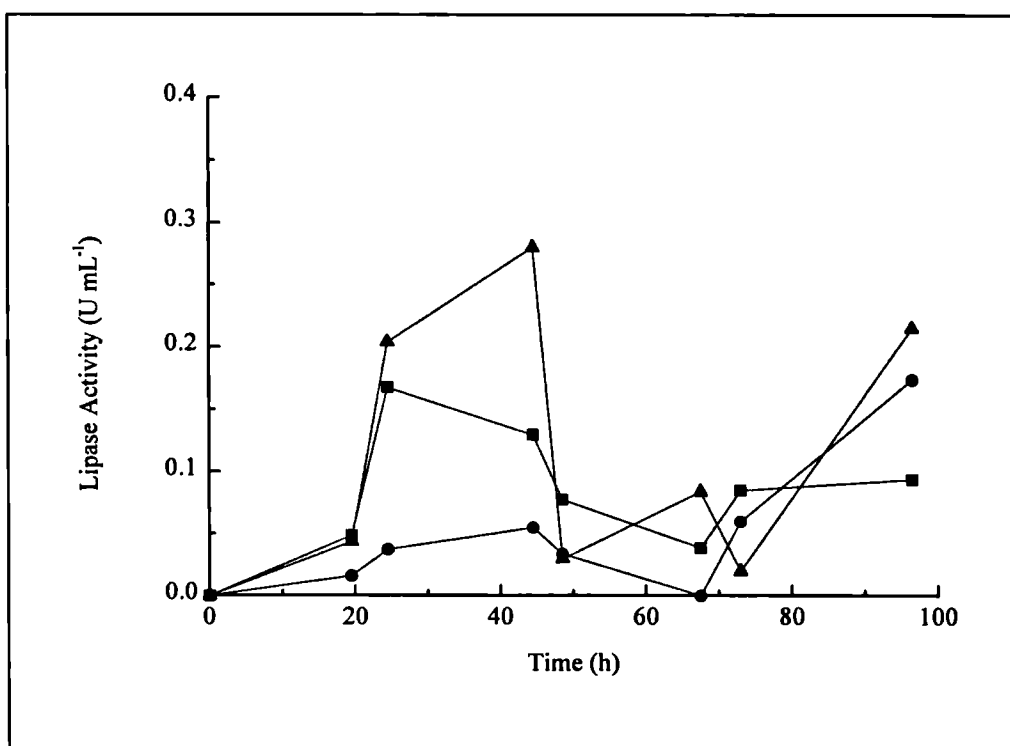
Lipase activity was measured using automatic titration against 0.01M NaOH, Section 3.3.1., using a tributyrin substrate. Specific lipase activity was calculated using viscosity as a measure of biomass levels. Viscosity has been used as a measure of specific activity due to a linear relationship with dry weight in previous *Streptomyces* fermentations (Sarraf *et al.*, 1996).

Triolein had similar lipase activity when compared to a rapeseed oil control, Figure 4.16.a. Given the biomass levels when triolein was used as a carbon source, Figure 4.15.a, lipase activity was as expected. Lipase activity, detected in the shake flask with tristearin as the carbon source, was lower than that of rapeseed oil, Figure 4.16.a. Biomass levels were approximately equal, Figure 4.15.a. Tristearin was solid at 26°C and this may have affected lipase activity. This may also be the case for the shake flasks containing tripalmitin as detected levels of activity were very low. Biomass levels were also lowest in the fermentations containing tripalmitin. The level of detected lipase activity may be low due to specificity of lipase activity in *S. clavuligerus* against tripalmitin. Methyl stearate enhanced lipase activity when compared to a rapeseed oil control and tristearin, Figure 4.16.b. Methyl oleate, triolein and rapeseed oil all have similar levels of lipase activity, Figure 4.16.c.

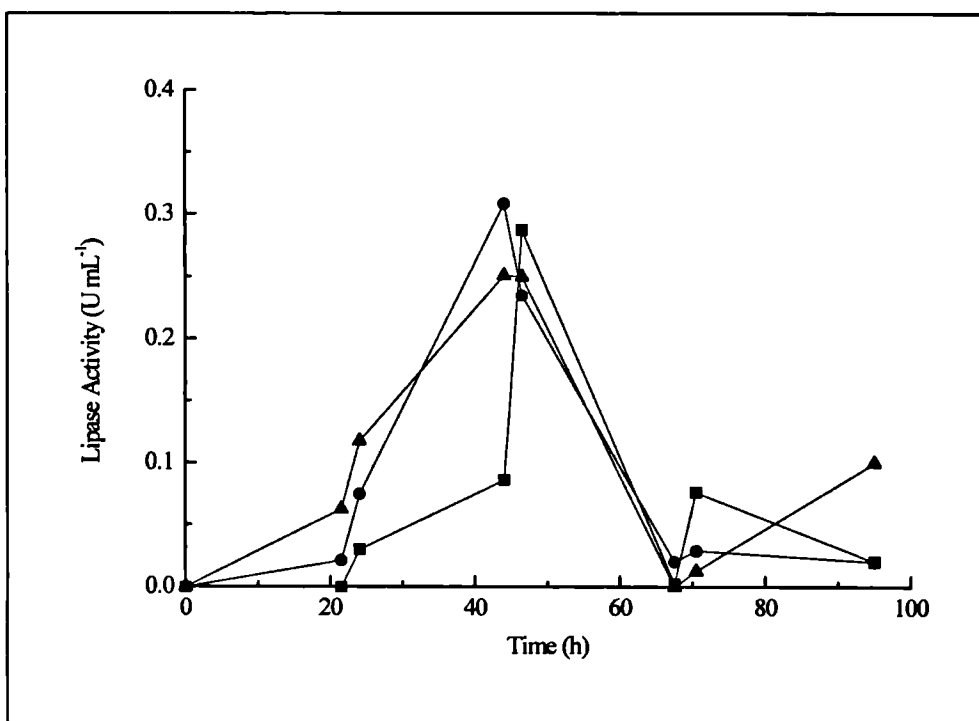
Specific lipase activities are shown in Figure 4.17. Specific activity takes in to account changes in biomass levels when discussing lipase activity. These data verify volumetric data in terms of the enhancement of lipase activity with methyl stearate and the similar levels of activities detected in fermentations containing methyl oleate, triolein and rapeseed oil.



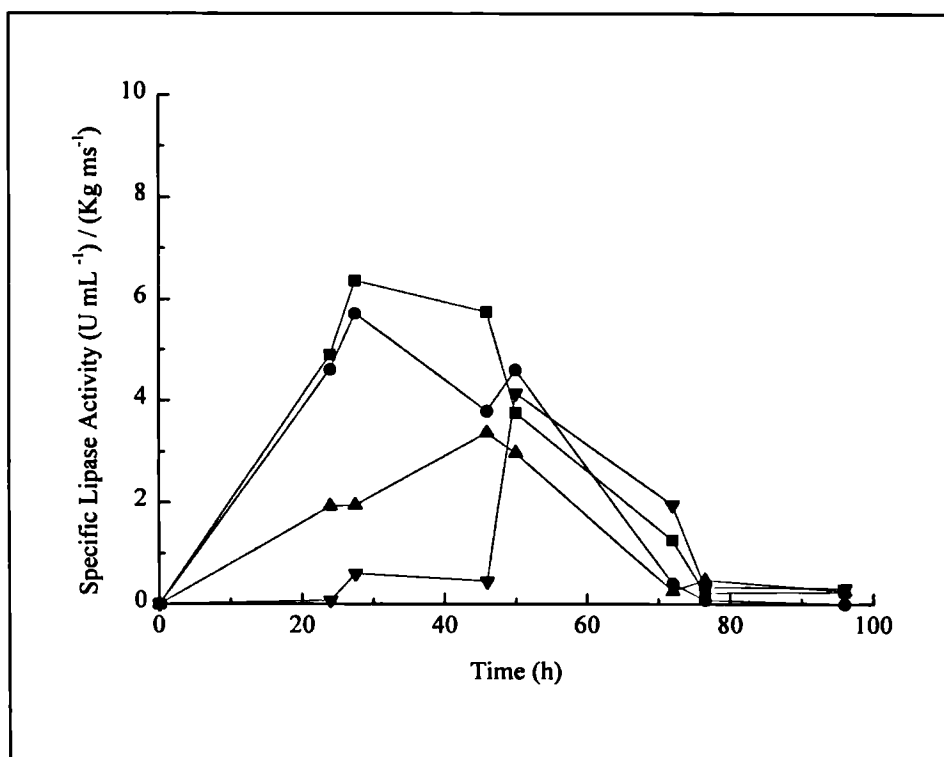
**Figure 4.16.a.** Lipase activity in shake flask fermentations containing rapeseed oil (■), triolein (●), tristearin (▲) and tripalmitin (▼).



**Figure 4.16.b.** Lipase activity in shake flask fermentations containing rapeseed oil (■), tristearin (●), and methyl stearate (▲).



**Figure 4.16.c. Lipase activity in shake flask fermentations containing rapeseed oil (■), triolein (●) and methyl oleate (▲).**



**Figure 4.17.a. Specific lipase activity in shake flask fermentations containing rapeseed oil (■), triolein (●), tristearin (▲) and tripalmitin (▼).**

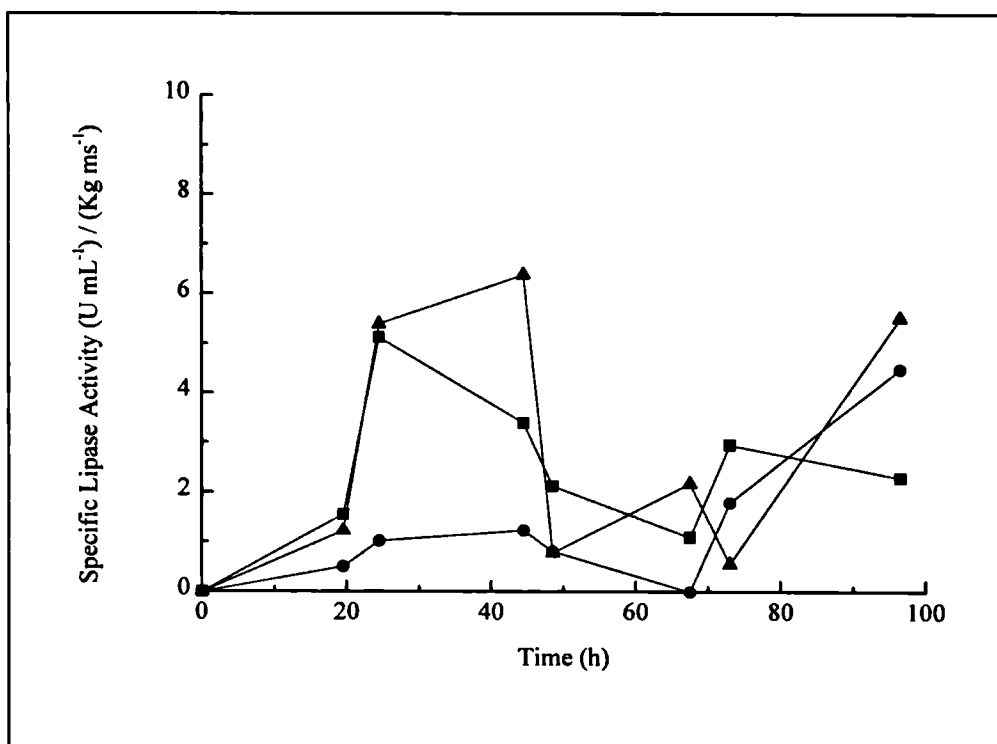


Figure 4.17.b. Specific lipase activity in shake flask fermentations containing rapeseed oil (■), tristearin (●), and methyl stearate (▲).

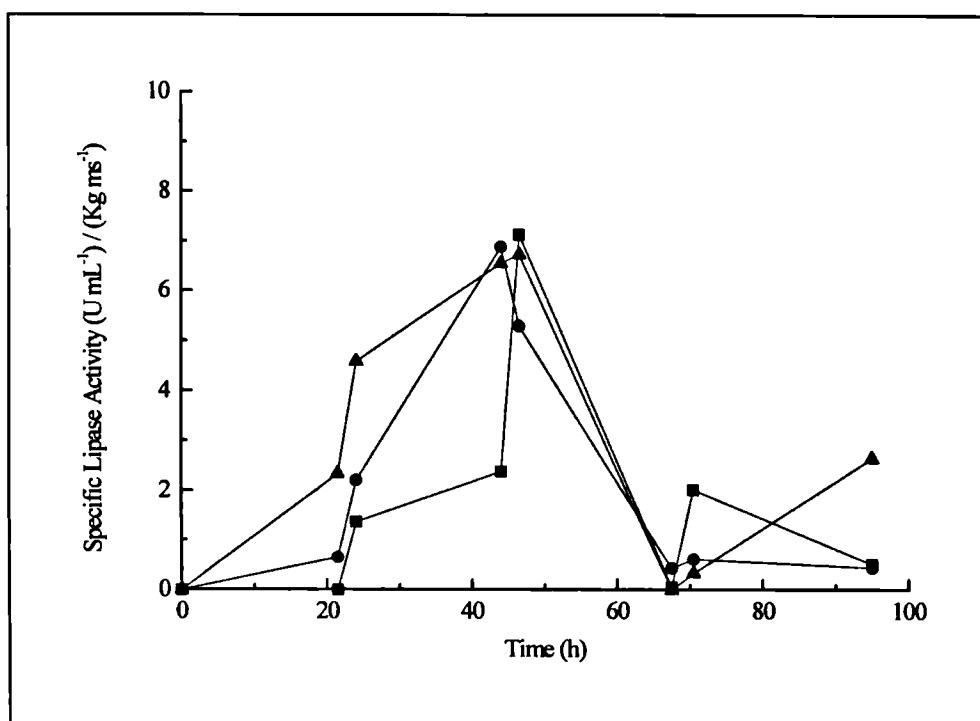


Figure 4.17.c. Specific lipase activity in shake flask fermentations containing rapeseed oil (■), triolein (●) and methyl oleate (▲).



#### 4.4.3. Discussion

These shake flask experiments aimed to determine any specificity with regards to utilisation of individual triacylglycerides present in rapeseed oil as this may contribute to residual oil levels. Determination of utilisation patterns may eliminate unused constituents and reduce residual oil levels. Such work is important to determine the contribution of components of rapeseed oil to growth and productivity of *S. clavuligerus*. Data presented in this study showed that triolein<sub>(C18:1)</sub> enhanced growth of *S. clavuligerus* when compared to a rapeseed oil control. Tristearin<sub>(C18:0)</sub> had no significant effect when compared to the control and tripalmitin<sub>(C16:0)</sub> reduced biomass levels. The triacylglyceride substrates had carbon contents of 75% (tripalmitin) and 77.2% (triolein and tristearin), differences that are probably not large enough to influence final biomass levels. It may be that *S. clavuligerus* has a preference for the unsaturated triolein over saturated lipid sources. The fatty acid methyl esters of oleic and stearic acid had no significant effect on growth measured by changes in broth viscosity in 2 L shake flask fermentations. Methyl oleate and methyl stearate demonstrated no toxic effects on growth of *S. clavuligerus*.

In a study into growth of different strains of *S. clavuligerus* on various oils and their fractions as main carbon sources (Lee and Ho, 1996) strains grew well on palm oil, palm kernal oil, their fractions and glycerol. Palm oil produced a higher dry cell weight than palm olein and palm stearin in one strain and no difference was seen in the other strain tested. Strains tested showed little or no growth on oleic acid yet growth on palm olein and palm kernal olein was significant. In the fermentation of *S. fradiae* on palm oil and its fractions for tylosin production palmitic acid was found to enhance growth whereas oleic acid inhibited when compared to palm olein, palm stearin and glycerol (Lee and Ho, 1997). Park *et al.* (1994) investigated whether the level of unsaturation of a fatty acid had an inhibitory effect on growth of *Streptomyces* sp. p6621. Linoleic<sub>(C18:2)</sub> and linolenic<sub>(C18:3)</sub> at levels higher than 0.25 g L<sup>-1</sup> inhibited growth, whereas oleic<sub>(C18:1)</sub> at 0.5 g L<sup>-1</sup> was inhibitory (Tsunoda, 1993). A strain resistant to high levels of increased saturation was necessary for improved growth.

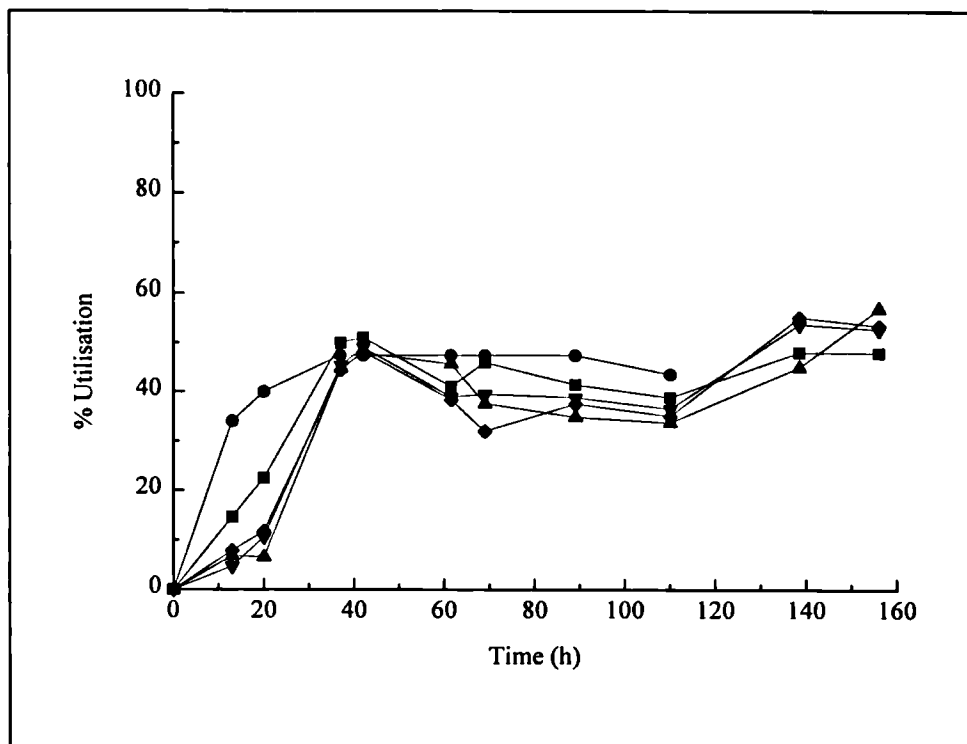
Lipase catabolises triolein to oleic acid and glycerol. Triolein was found to enhance growth of *S. clavuligerus* in this study. As lipase activity in *S. clavuligerus* has been reported to be associated with the cell membrane (Cavanagh, 1996) the oleic acid may

be transported directly into the cell after release from the triacylglyceride at the cell surface and immediately acyl CoA added inside the cell membrane to detoxify. The fatty acid and acyl CoA complex then enter the  $\beta$ -oxidation cycle. Fatty acid methyl esters were found not to have any significant toxic effects on growth of *S. clavuligerus* in this study. Methyl esters may be transported into the cell intact or demethylated at the surface of the cell and transported inside the organism for immediate detoxification with acyl CoA. Triacylglycerides and fatty acid methyl esters of chain length C3 to C22 made better carbon sources for growth of *S. lividans* than free fatty acids with no observed differences in odd and even carbon numbers (Peacock, 1998). It can be concluded that growth of different strains and genera of Streptomyces may be enhanced by specific lipids or their constituents when used as a carbon source, the mechanism of which is still unclear.

A gas chromatography method was developed, Section 3.4., in order to determine any preferential utilisation of the constituents of rapeseed oil used in the complex process media. The results for individual fatty acids are compared to total lipid analysis, Figure 4.18. Chromatographic data showed no selectivity in terms of the fatty acid constituent of rapeseed oil. The residual oil level is therefore of similar composition to the initial rapeseed oil.

Of the triacylglycerides tested in this study triolein had similar lipase activity when compared to rapeseed oil, tristearin less and tripalmitin showed a significant decrease in detected lipase activity. Methyl stearate enhanced lipase activity when compared to a rapeseed oil control. Previous studies have shown that the type of oil used in fermentation affects induction of lipase activity in many different organisms (Sztajer *et al.*, 1989). After an extensive lipolytic screening program Sztajer *et al.* (1989) found lipase activity dramatically increased in many organisms with the addition of olive oil when compared to shorter chain triacylglycerides such as tributyrin. Lipase activity in different organisms is induced by variations in oil content. It may be that lipase activity in *S. clavuligerus* is induced by the specific chain length or the molecular configuration of methyl stearate. Specific lipase activity may be lowest in tripalmitin due to the physical state of the substrate, that is as flakes of approximately 3mm<sup>2</sup>, or the reduced chain length. It may be concluded that lipase activity in different strains and genera of

Streptomycetes may be increase or inhibited by specific lipids or their constituents when used as a carbon source. The mechanism of enhancement or inhibition is of yet unclear.



**Figure 4.18. Gas chromatography data showing the utilisation of fatty acids throughout 5 L batch fermentation of *S. clavuligerus*.**

***Palmitic (■), Stearic (●), Oleic (▲), Linoleic (▼) and Linolenic (◆).***

No clavulanic acid was detected in the shake flask experiments containing complex media due to the scale of fermentation. Variations in lipid substrate have been reported to affect antibiotic titres. The effects of various oils on cephamycin C production in fermentation of *Cephalosporium acremonium* (Paul *et al.*, 1997) concluded sesame oil, which is high in oleic and linoleic acid, increased productivity. Methyl oleate is often used in a culture media to increase cephalosporin C production (Nash *et al.*, 1985). Lee and Ho (1997) reported that the use of long chain saturated fatty acids resulted in high tylosin production from *S. fradiae*. Glycerol, palmitic and stearic acids enhanced production. No tylosin was detected when oleic acid was used as the carbon source. Utilisation of pure glycerol, palmitic and stearic acids, available from oleochemical plants, would avoid none utilisation of oleic acid with palm oil feed stock. In the study of clavulanic acid production from *S. clavuligerus* (Lee and Ho, 1996) Palm oil provided the best carbon source for production although use of palm oil fractions also

produced considerable clavulanic acid. Palmitic and stearic acids produced little clavulanic acid and no production was observed using sucrose. Further investigation in this study into the use of various triacylglycerides, methyl esters and fatty acids on a 5 L pilot plant scale was not possible due to the cost of raw materials. Data would be of interest in terms of enhancement of clavulanic acid titres and elimination of any inhibitory effects due to the mixture of fatty acids present in rapeseed oil. Specificity for individual fatty acids and triacylglycerides in terms of enhancing productivity would be of further interest.

#### **4.5. Summary of the effect of media composition and pH**

To achieve optimal process control in 5 L batch fermentations of *S. clavuligerus* pH must be controlled using both acid and base. An initial rise in pH affects both growth and lipase activity in 5 L batch fermentations of *S. clavuligerus* containing complex media.

Addition of rapeseed oil, as part carbon source in combination with modified starch, increased final clavulanic acid titres by 900% in the strain of *S. clavuligerus* used. This may not be the case in the current production strain.

Type and concentration of soyaflour used in the process media affected lipase activity. An increase in concentration of flour 1 increased lipase activity. An increase in flour 2 decreased detected lipase activity. The difference was attributed to protein levels within the flour.

The concentration of soya flour affected initial rates of oil utilisation. A decrease in flour from 3:1 to 2:1 flour:modified starch increased initial utilisation rates. The difference may be due to particle size in the fermenter environment affecting dispersion of the lipid substrate. Increased dispersion may increase rapeseed oil utilisation. Residual oil levels remained high regardless of soyaflour type and concentration, at approximately 30% of the initial oil concentration.

When comparing separate triacylglycerides it was found triolein enhanced growth when compared to rapeseed oil, tristearin and tripalmitin in shake flask experiments containing complex media. Methyl stearate enhanced detected lipase activity when compared to tristearin and rapeseed oil in complex media.

Gas chromatography analysis of fermentation broth samples revealed no specificity in terms of fatty acid utilisation. It was noted that as the method used to analyse the constituents of rapeseed oil breaks down the triacylglycerides to fatty acids, specificity in relation to positions of fatty acids within a triacylglyceride cannot be determined.

## **5. THE EFFECT OF AGITATION RATE ON LIPID UTILISATION AND CLAVULANIC ACID PRODUCTION IN *S. CLAVULIGERUS***

Research has been undertaken to examine the effects of impeller tip speed on lipid utilisation and clavulanic acid production in 5 L batch cultivation of *S. clavuligerus*. Due to the particulate nature of the complex process media, and the hyphal morphology of *S. clavuligerus*, methods of traditional biomass measurement, dry weight and optical density, were not possible. Changes in viscosity and capacitance of the fermentation media were used in order to compare the effect of tip speed on cell concentration. Image analysis was used to examine changes in hyphal morphology. Lipase activity and specific oil utilisation rates are also reported. Residual oil levels and the percentage utilisation of individual triacylglycerides present in rapeseed oil were also studied. A comparison was made between clavulanic acid titres in all fermentations carried out.

The tip speeds used covered the entire range practical for the 5 L pilot scale bioreactor, LH2000, Figure 3.1. The highest value,  $3.77 \text{ m s}^{-1}$ , being close to that used on a typical industrial production scale. Power per unit volume is shown in Table 5.1. An additional fermentation was carried out to investigate the effect of stepped changes in tip speed during the process. Tip speed ramping during process might be useful in minimising the power input required over the fermentation cycle if high shear rates limit mycelial branching and increase hyphal fragmentation, without adverse productivity impact.

**Table 5.1. Power per unit volume for agitation rates used**

Agitation rate (rpm)	Tip speed ( $\text{m s}^{-1}$ )	Pug (W)	Pg (W)	Pg/V ( $\text{W m}^{-3}$ )
600	1.88	10.5	4.0	805.3
750	2.36	20.5	8.1	1625.7
900	2.83	35.4	14.4	2885.3
1200	3.77	84.0	35.7	7146.2

In all fermentations the level of dissolved oxygen was maintained above 50% using an air flow rate of 1 vvm or, where necessary, by blending oxygen with the inlet air. Where possible the fermentations were carried out in duplicate and average results shown.

## **5.1. The effect of tip speed on growth and morphology**

Results of growth and morphology in batch cultivation of *S. clavuligerus* on a 5 L scale at tip speeds of 1.88 m s<sup>-1</sup>, 2.36 m s<sup>-1</sup>, 2.83 m s<sup>-1</sup> and 3.77 m s<sup>-1</sup> are described. Media viscosity and the change in the capacitance of the media during the process have been shown to correlate with dry weight in previous fermentations using filamentous organisms (Sarra *et al.*, 1996) and were therefore used as a measure of changes in biomass concentration. Image analysis was used as a tool to study the changes in hyphal morphology of *S. clavuligerus* within the fermenter environment.

### **5.1.1. Inocula preparation**

Inocula for all fermentations were prepared in 2 x 2 L shake flasks containing 200 mL of seed medium, Section 3.1.7. Any noted differences in initial growth rates or biomass concentration were not due to seed transfer as this was considered a critical process parameter and monitored closely. The incubation time, broth viscosity and pH of each seed were noted before transfer. If any significant deviations were observed in both shake flasks seed transfer did not take place and the inocula preparation repeated.

### **5.1.2. Dissolved oxygen tension (DOT)**

An air flow rate of 1 vvm was found to provide enough oxygen to maintain the DOT above 50% in the majority of fermentations carried out. Where necessary the DOT was further maintained above 50% by blending oxygen into the air feed whilst maintaining a constant air flow rate. The maintenance of DOT above 50% was necessary in order to eliminate the effects of low oxygen concentration on results as this has been shown to affect Streptomyces growth (Yegneswaran *et al.*, 1991a and 1991b), productivity in *S. clavuligerus* (Rollins *et al.*, 1990) and oil consumption in *S. fradiae* (Chen and Wilde, 1991).

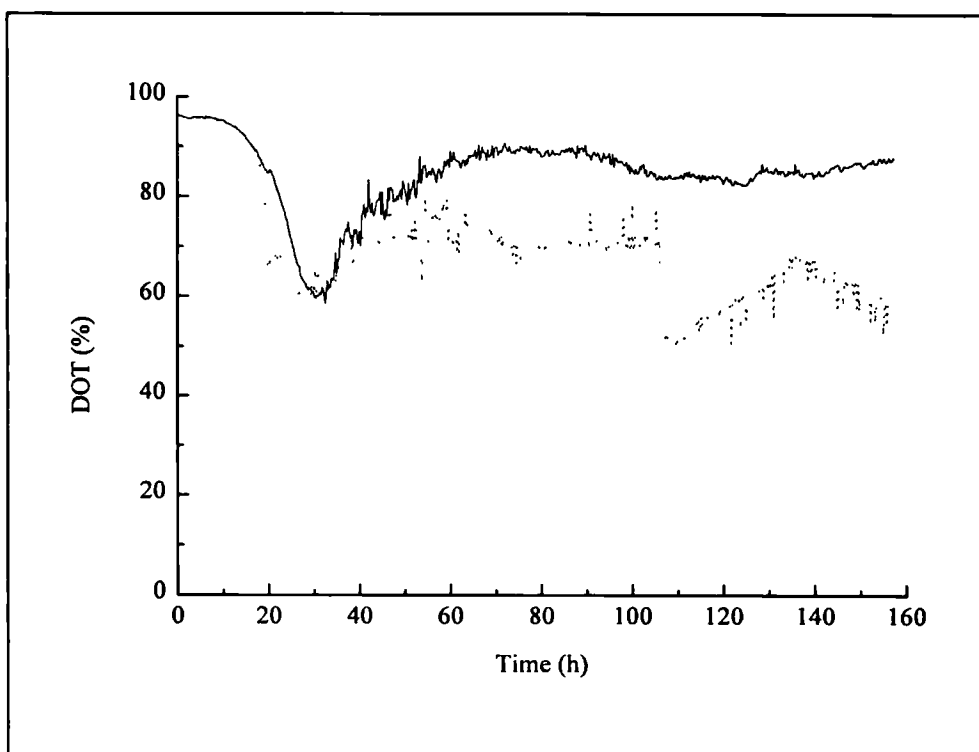
The DOT profiles for all tip speed comparison fermentations are shown in Figure 5.1. None of the DOT traces show a prominent lag phase due to correct transfer of the seed culture to the fermenter environment whilst in rapid growth phase.

Figure 5.1.a. shows the level of DOT during the course of the fermentations held at constant tip speeds of  $1.88 \text{ m s}^{-1}$  and  $2.36 \text{ m s}^{-1}$ . The DOT profiles show good homology, there is no significant difference between fermentations in the initial stages, 0-100 h. The DOT fell in the initial 40 h then slowly increased as the organism ceases to require the total amount of oxygen being supplied and enters the stationary phase of the cell cycle. The fall in DOT towards the end of a fermentations held at  $2.36 \text{ m s}^{-1}$  and  $2.83 \text{ m s}^{-1}$  could represent a secondary growth phase or correlate to changes in organism morphology.

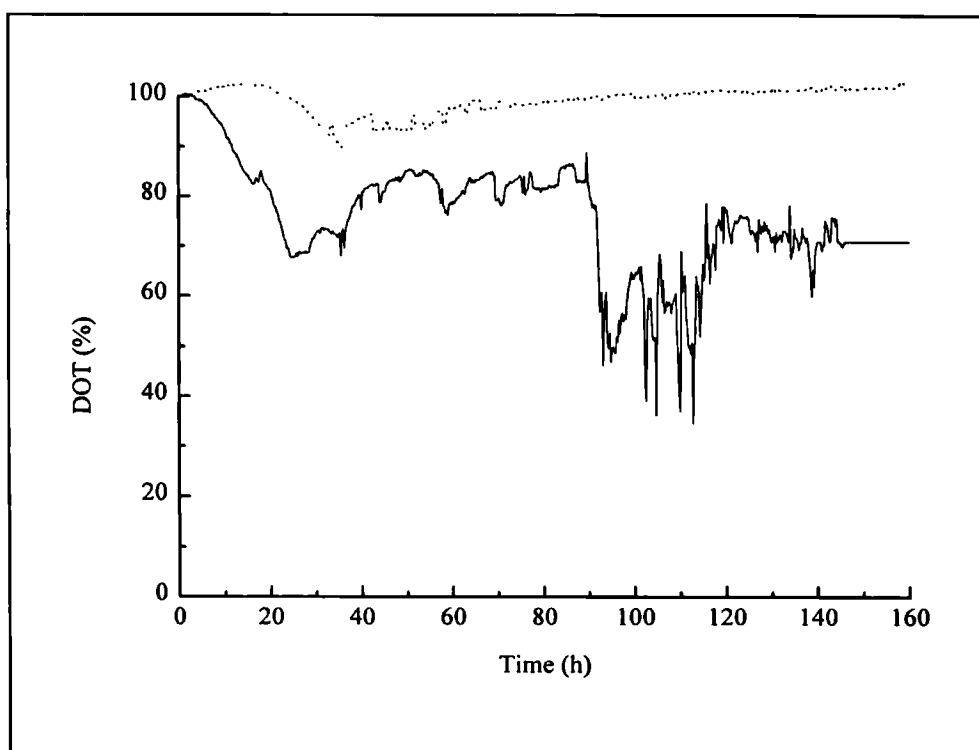
DOT profiles for the fermentations maintained at tip speeds of  $2.83 \text{ m s}^{-1}$  and  $3.77 \text{ m s}^{-1}$ , Figure 5.1.b, show that at  $2.83 \text{ m s}^{-1}$  the initial fall in DOT was more rapid than that of the fermentation held at  $3.77 \text{ m s}^{-1}$ . This indicates a more rapid initial growth phase. Differences in initial growth rates were not due to differences in inocula as all seed transfer were monitored closely. A decrease in DOT, at  $2.83 \text{ m s}^{-1}$ , at 80-100 h, was similar to that seen and described in the fermentation held at  $2.36 \text{ m s}^{-1}$ . The DOT for the fermentation held at  $3.77 \text{ m s}^{-1}$  did not fall below 90% air saturation;  $3.77 \text{ m s}^{-1}$  is at the upper limit of tip speed used on a 5 L scale. Higher tip speed maintains a high oxygen transfer environment.

Figure 5.1.c. shows a profile of DOT during which the tip speed was increased from  $2.83 \text{ m s}^{-1}$  to  $3.77 \text{ m s}^{-1}$  after 48 h cultivation. Tip speed was increased in order to minimise detrimental affects of shear during initial growth. DOT decreased, 0-40 h, and then increased as the cells enter stationary phase. After 48 h, the point at which the tip speed was increased, the DOT remains high until 130 h due to the increase in oxygen transfer. A decrease in DOT is seen after 130 h as in the fermentations held at  $2.36 \text{ m s}^{-1}$  and  $2.83 \text{ m s}^{-1}$ .

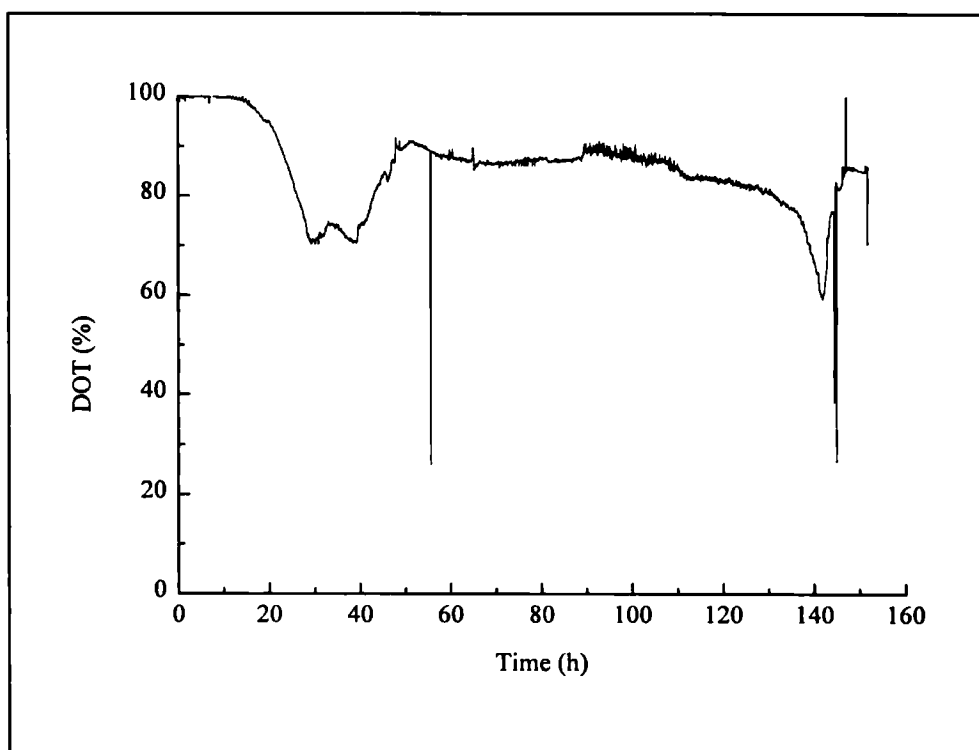




**Figure 5.1.a.** DOT measurements during fermentations at tip speeds of  $1.88 \text{ m s}^{-1}$  ( - ) and  $2.36 \text{ m s}^{-1}$  ( --- )



**Figure 5.1.b.** DOT measurements during fermentations at tip speeds of  $2.83 \text{ m s}^{-1}$  ( - ) and  $3.77 \text{ m s}^{-1}$  ( --- )



**Figure 5.1.c. DOT measurements during a fermentation where tip speed increased from  $2.83 \text{ m s}^{-1}$  to  $3.77 \text{ m s}^{-1}$  at 48 h**

### 5.1.3. Viscosity

Changes in biomass concentration were represented by measurements of broth viscosity using a Brookfield viscometer, Section 3.2.1. Broth viscosity throughout the course of the fermentations held at constant and stepped stirrer speeds are shown in Figure 5.2.

Figure 5.2.a. showed that the batch fermentations carried out at constant tip speeds have an approximate 20 h delay in change in viscosity which may correlate to a short lag phase. On-line DOT data did not show a significant lag phase, Figure 5.1. The initial stages of growth which affect the DOT would not affect media viscosity as the cell concentration is very low. As the cell concentration increases changes in biomass can be detected using viscosity. At tip speeds of  $1.88 \text{ m s}^{-1}$ ,  $2.36 \text{ m s}^{-1}$  and  $2.83 \text{ m s}^{-1}$  maximum biomass levels were reached after 40 h. At a tip speed of  $2.83 \text{ m s}^{-1}$  the growth phase appears extended and the decline in growth more gradual. It can be postulated that at this tip speed hyphal growth exceeds hyphal breakage. A tip speed of  $3.77 \text{ m s}^{-1}$  showed an extension in the lag phase, a slower initial growth rate and a decrease in maximum viscosity when compared to the fermentations held at tip speeds

of  $2.36 \text{ m s}^{-1}$  and  $2.83 \text{ m s}^{-1}$ . This may be due to the shear forces induced by the increased tip speed affecting hyphal extension and therefore broth viscosity.

Viscosity data shown in Figure 5.2.b. represents changes in biomass concentration throughout the fermentation where the tip speed was increased from  $2.83 \text{ m s}^{-1}$  to  $3.77 \text{ m s}^{-1}$  after 48 h cultivation. At a constant tip speed of  $3.77 \text{ m s}^{-1}$ , viscosity is lower than at  $2.83 \text{ m s}^{-1}$ , Figure 5.2.a. This is thought to be due to changes in hyphal morphology and increased hyphal fragmentation induced by the increase in shear forces. When the tip speed was increased during the course of the fermentation the viscosity remained high. The higher viscosity is an indication that fragmentation is reduced. This may be due to the fact that the hyphae in stationary phase are stronger and more resistant to shear forces than pre-stationary hyphae.

This theory is supported by research into hyphal morphology of *Saccharopolyspora erythraea* (Heydarian, 1998). On homogenisation of samples of different ages throughout fermentation under various tip speeds, protein release from *S. erythraea* decreased with increased fermentation time. The value of  $K_p$  in the Hetherington equation (Hetherington *et al.*, 1971), a dimensionless constant dependent on physio-chemical properties of the cell wall and disruptive pressure, decreased throughout the process. This indicated that a sample from a later time in fermentation had a lower value of  $K_p$  which may be an indication of increased cell wall strength. Values of  $K_p$  for fermentations held at higher tip speeds, same fermentation time, were lower when compared to lower tip speeds. A decrease in mycelial size decreased the effect of disruptive forces and an increase in cell wall strength increased the effect of constructive forces either or both of which may be responsible for a decrease in  $K_p$ .

This may be explained as it has been reported that hyphal cell walls in actinomycetes form gradually by polymerisation starting at the hyphal tip and continuing some distance behind (Miguel *et al.*, 1993) a consequence of which is that hyphal wall thickness depends on extent of polymerisation. The hyphae become weakest near the tip and become thicker with increasing distance from the tip.

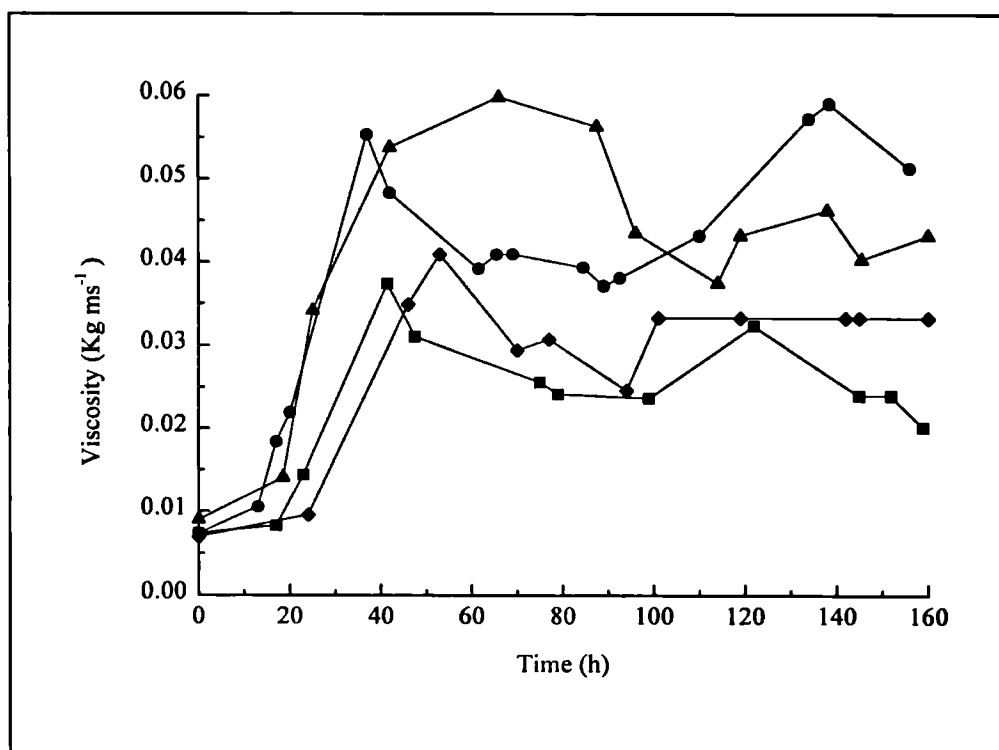


Figure 5.2.a. Viscosity measurements for a *S. clavuligerus* batch cultivation at tip speeds of 1.88 m s<sup>-1</sup> (■), 2.36 m s<sup>-1</sup> (●), 2.83 m s<sup>-1</sup> (▲) and 3.77 m s<sup>-1</sup> (◆)

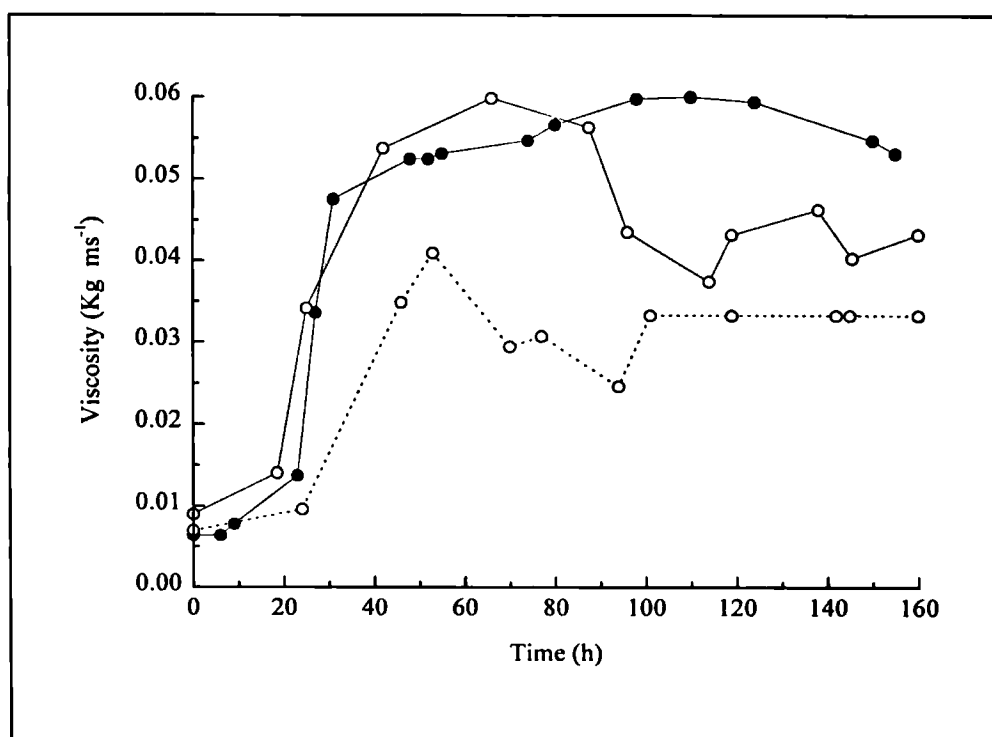


Figure 5.2.b. Viscosity measurements for a *S. clavuligerus* fermentation held at tip speeds of 2.83 m s<sup>-1</sup> (○), 3.77 m s<sup>-1</sup> (◌) and 2.83 m s<sup>-1</sup> to 3.77 m s<sup>-1</sup> at 48 h (●)

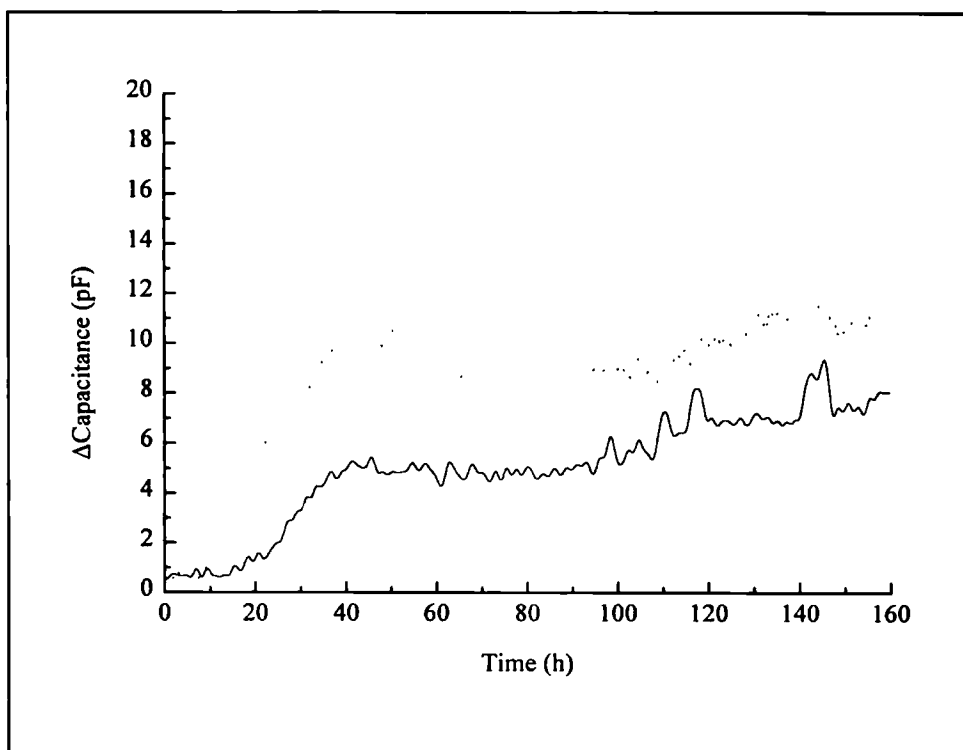
#### 5.1.4. Capacitance

The biomass monitor was used as an on-line tool for measuring changes in cell concentration within the bioreactor, Section 3.2.2. The basis of the biomass probe is the measurement of changes in the amount of membrane polarisation at a set frequency, i.e. the change in media capacitance. The relationship between biomass concentration, determined by the capacitance based probe, rheology and morphology have been successfully investigated in cultures of *Saccharopolyspora erythraea* in 5 L batch fermentations (Sarraf *et al.*, 1996). Advantages of this technique over viscosity include the ability to monitor the culture on-line and that the biomass probe is able to separate cell debris from viable cells since only intact viable membranes contribute to the capacitance measured.

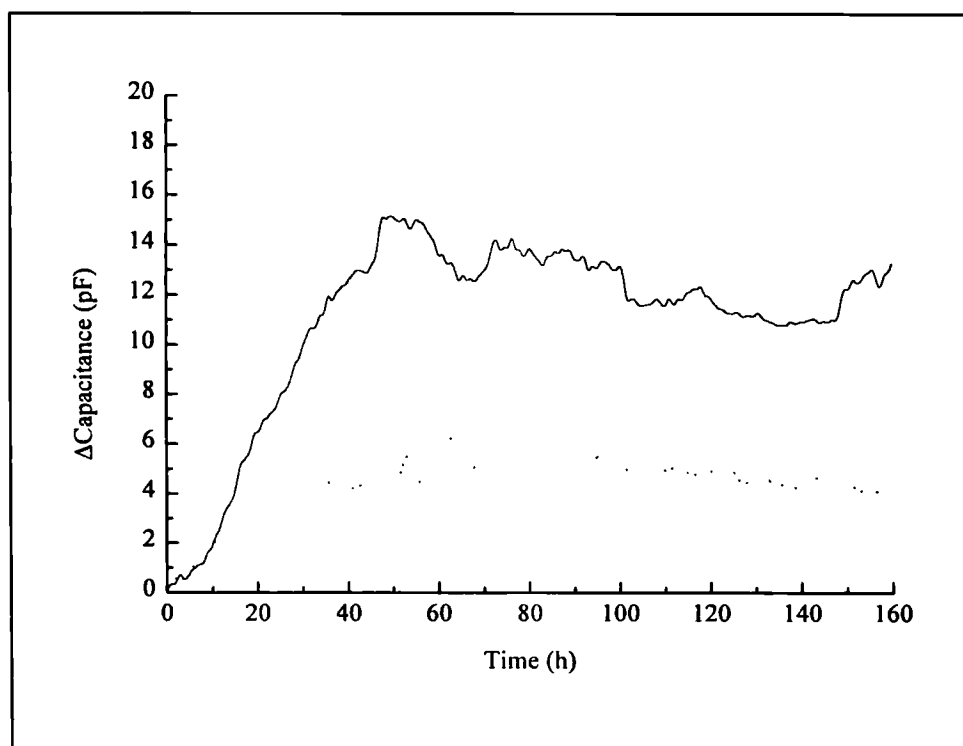
Variability in capacitance due to increased tip speed is accounted for by initial offset calibration of the biomass monitor. The capacitance results are shown in Figure 5.3. The data shown was smoothed to reduce on-line noise using FFT filter (Microcal Origin).

The profiles for constant tip speed, Figures 5.3.a. and 5.3.b., show an increase in capacitance with an increase in tip speeds from  $1.88 \text{ m s}^{-1}$  to  $2.83 \text{ m s}^{-1}$ . The results indicate that the maximum capacitance measured occurs at 45-50 h into the fermentation. At the tip speed of  $1.88 \text{ m s}^{-1}$  the maximum capacitance reached at 40 h was approximately 5.5 pF, at  $2.36 \text{ m s}^{-1}$  a capacitance of 11 pF was reached. When the tip speed was increased to  $2.83 \text{ m s}^{-1}$  the maximum capacitance reached was approximately 15 pF. The maximum capacitance reached at  $3.77 \text{ m s}^{-1}$  was 6.5 pF. The decrease in capacitance at the highest tip speed indicates a decrease in polarisable membranes. This may be due to fragmentation of the hyphal cell walls by increased shear forces induced by such a high impeller tip speed. The theory of increased fragmentation or changes in hyphal morphology is supported by the decrease in the broth viscosity.

Changes in on-line capacitance of the process media correlate well with the viscosity measurements used to measure changes in biomass concentration, Figure 5.4.

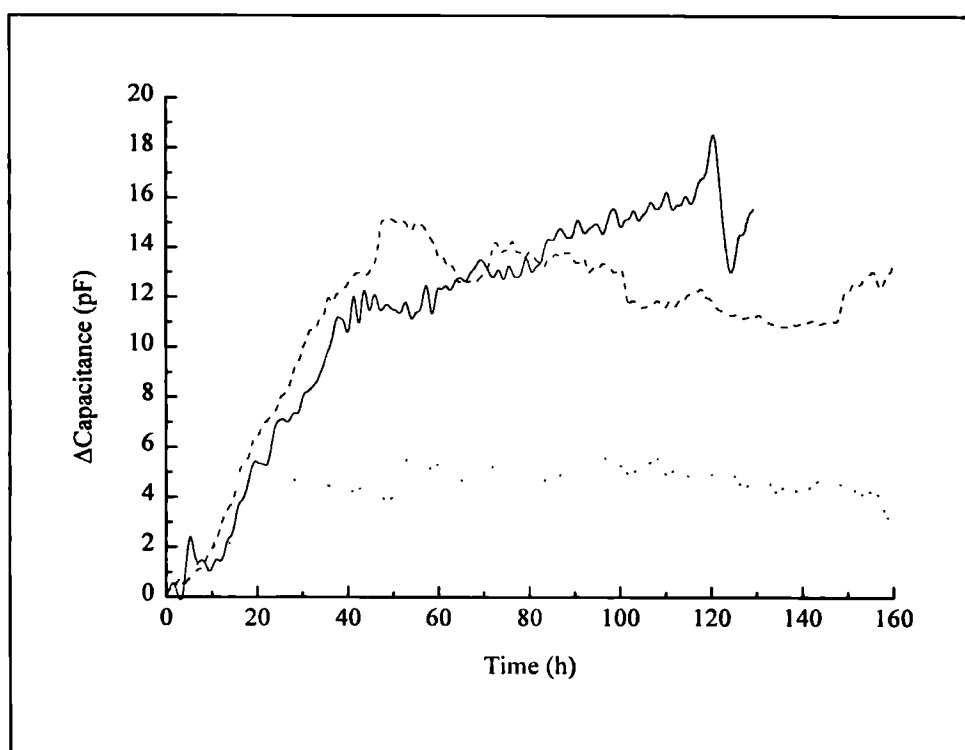


**Figure 5.3.a.** On-line  $\Delta$ capacitance measurements during a batch cultivation at tip speeds of  $1.88 \text{ m s}^{-1}$  ( - ) and  $2.36 \text{ m s}^{-1}$  ( --- )

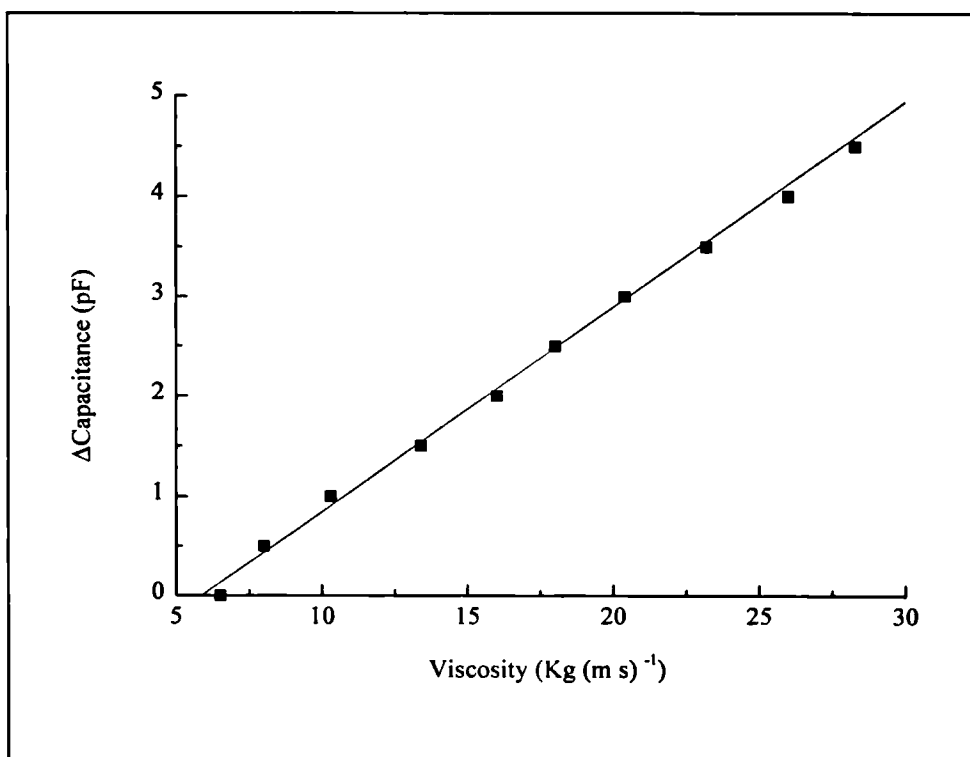


**Figure 5.3.b.** On-line  $\Delta$ capacitance measurements during a batch cultivation at tip speeds of  $2.83 \text{ m s}^{-1}$  ( - ) and  $3.77 \text{ m s}^{-1}$  ( --- ).

The changes in cell concentration for the stepped tip speed fermentation are shown in Figure 5.3.c. The initial change in capacitance for the stepped tip speed fermentation follows that of the constant tip speed as expected. The capacitance measured for the fermentation held at  $3.77 \text{ m s}^{-1}$  was very low, attributed to an increase in hyphal fragmentation. When the tip speed was increased during the fermentation the capacitance remained high. The change in capacitance was not due to the increase in stirrer speed as this was accounted for in offset recalibration of the biomass probe at 48 h. The higher difference in capacitance supports the theory that the hyphae are more resistant to hyphal fragmentation after 48 h cultivation than if exposed to high shear initially. The final capacitance readings, 120-160 h, are not complete due to hyphal clogging of the biomass probe pins.



**Figure 5.3.c. On-line  $\Delta$ capacitance measurements during a batch cultivation at tip speeds of  $2.83 \text{ m s}^{-1}$  (---) and  $3.77 \text{ m s}^{-1}$  (.....) and  $2.83 \text{ m s}^{-1}$  to  $3.77 \text{ m s}^{-1}$  (—)**



**Figure 5.4. Correlation of on-line  $\Delta$ capacitance and off-line broth viscosity.**

#### 5.1.5. Exit gas analysis

Exit gas data collection is incomplete due to mechanical error in the gas mass spectrometry and data acquisition.

The maximum OUR reached in the fermentation held at a tip speed of  $1.88 \text{ m s}^{-1}$  was approximately  $12 \text{ mmol L}^{-1} \text{ h}^{-1}$  at 30 h, with a maximum CER value of  $8.5 \text{ mmol L}^{-1} \text{ h}^{-1}$ . Maximum OUR values in the initial stages of growth during the fermentation held at  $2.36 \text{ m s}^{-1}$  increased to approximately  $18 \text{ mmol L}^{-1} \text{ h}^{-1}$  at 22 h. Initial CER and OUR data were not collected from the fermentation at  $2.83 \text{ m s}^{-1}$  due to data logging failure. The maximum OUR value in the fermentation held at  $3.77 \text{ m s}^{-1}$  was similar to previous fermentations at approximately  $17.5 \text{ mmol L}^{-1} \text{ h}^{-1}$  at 40 h. Although measurements of cell concentration, using changes in media viscosity and capacitance, were low for the fermentation held at  $3.77 \text{ m s}^{-1}$  exit gas data suggests substantial growth took place. The differences in media viscosity and capacitance at the different tip speeds may not be due to the difference in rates of change in cell growth or biomass concentration but to differences in hyphal morphology.



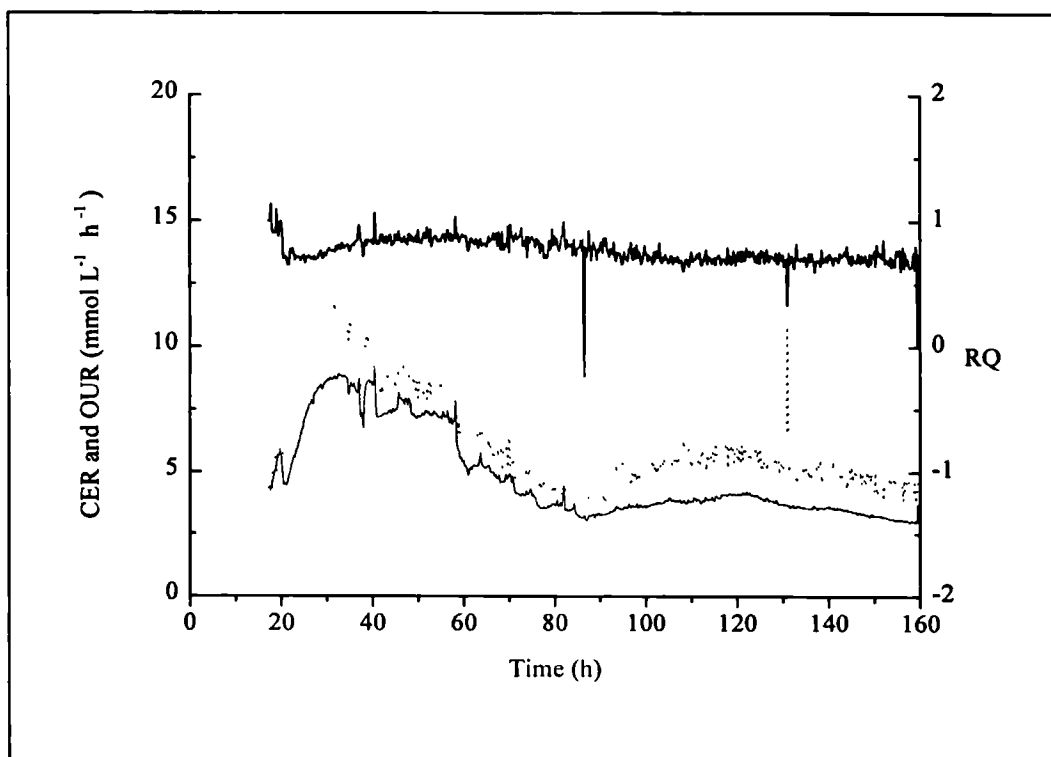


Figure 5.5.a. Exit gas analysis for fermentation held at  $1.88 \text{ m s}^{-1}$ .

( --- OUR — CER, — RQ )

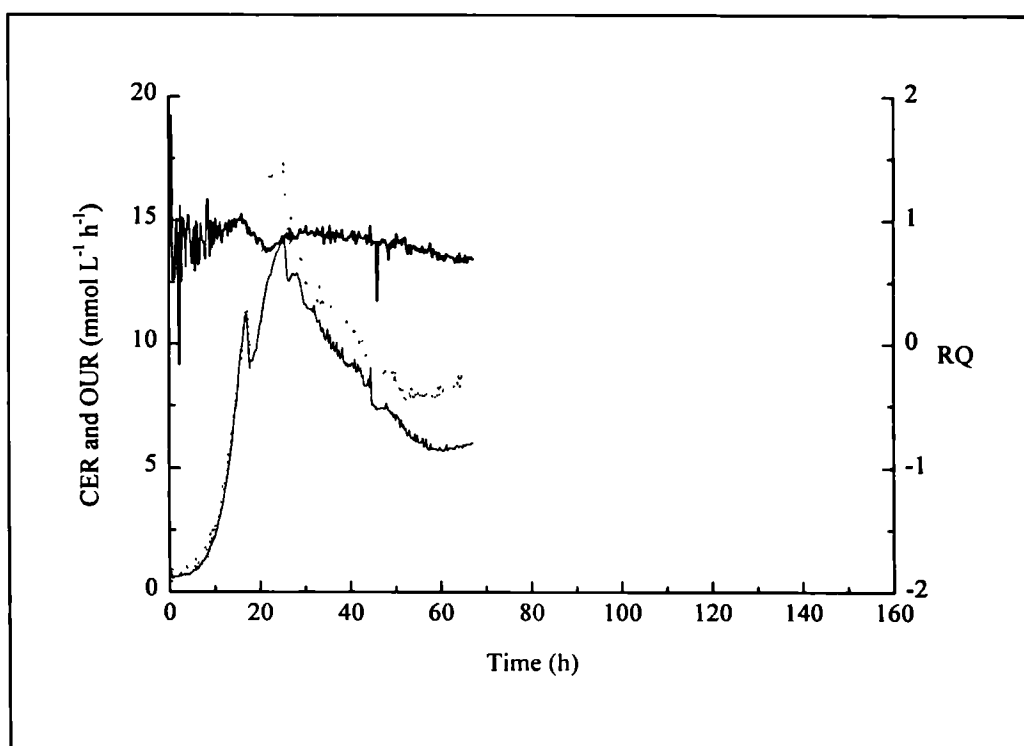


Figure 5.5.b. Exit gas analysis for fermentation held at  $2.36 \text{ m s}^{-1}$ .

( --- OUR — CER, — RQ )

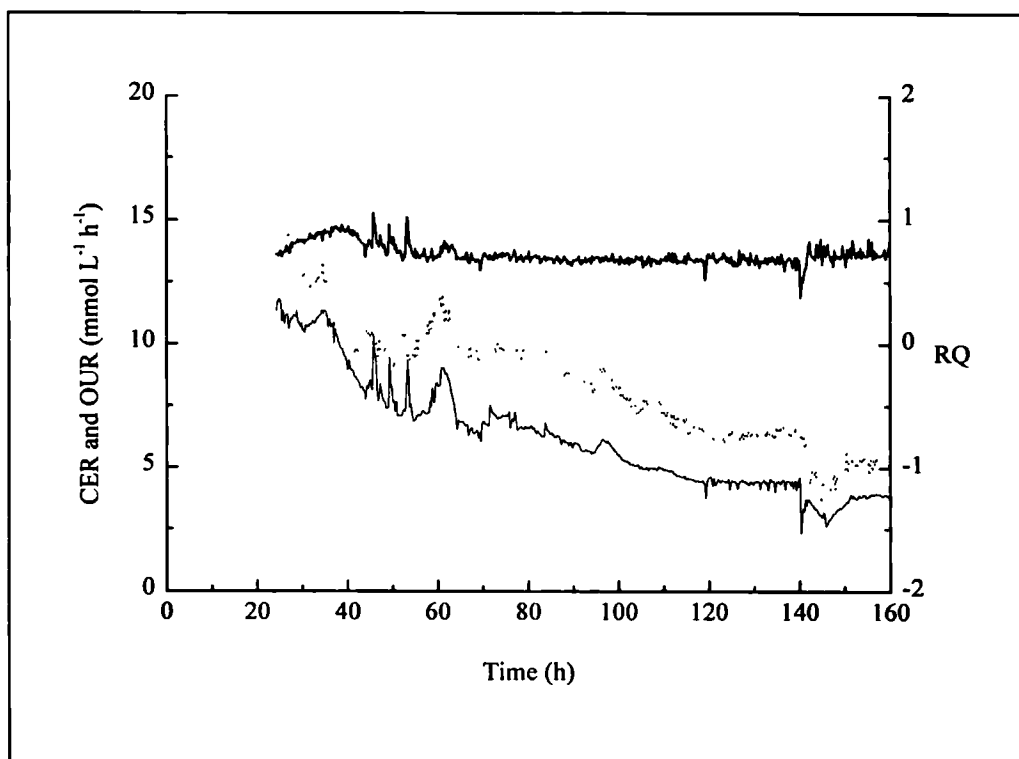


Figure 5.5.c. Exit gas analysis for fermentation held at  $2.83 \text{ m s}^{-1}$ .

( --- OUR — CER, — RQ )

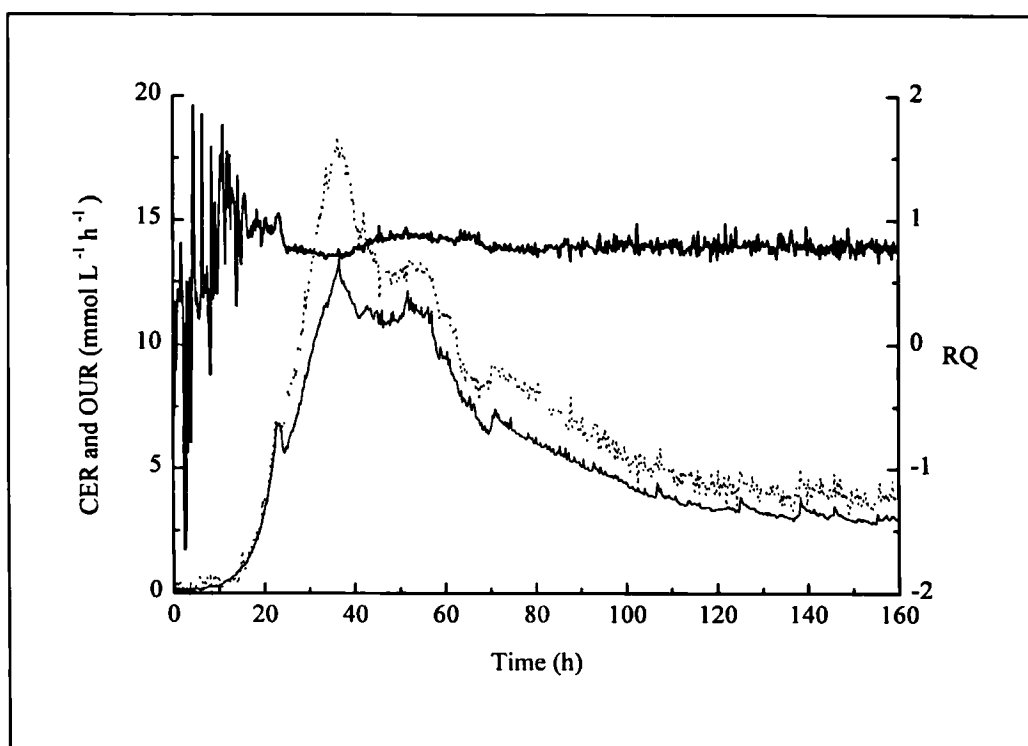


Figure 5.5.d. Exit gas analysis for fermentation held at  $3.77 \text{ m s}^{-1}$ .

( --- OUR — CER, — RQ )

### 5.1.6. The effect of tip speed on hyphal morphology

#### 5.1.6.1. Microscopic observations

Initial microscopic observations revealed a significant difference in hyphal lengths between the fermentations held at tip speeds of  $2.83 \text{ m s}^{-1}$  and  $3.77 \text{ m s}^{-1}$ , Figure 5.6. These differences were quantified with the use of image analysis, 5.1.6.2.



**Figure 5.6.a.** Hyphal development after 72 h fermentation at  $2.83 \text{ m s}^{-1}$ .



**Figure 5.6.b. Hyphal development after 72 h fermentation at 3.77 m s<sup>-1</sup>.**

#### 5.1.6.2. Image analysis

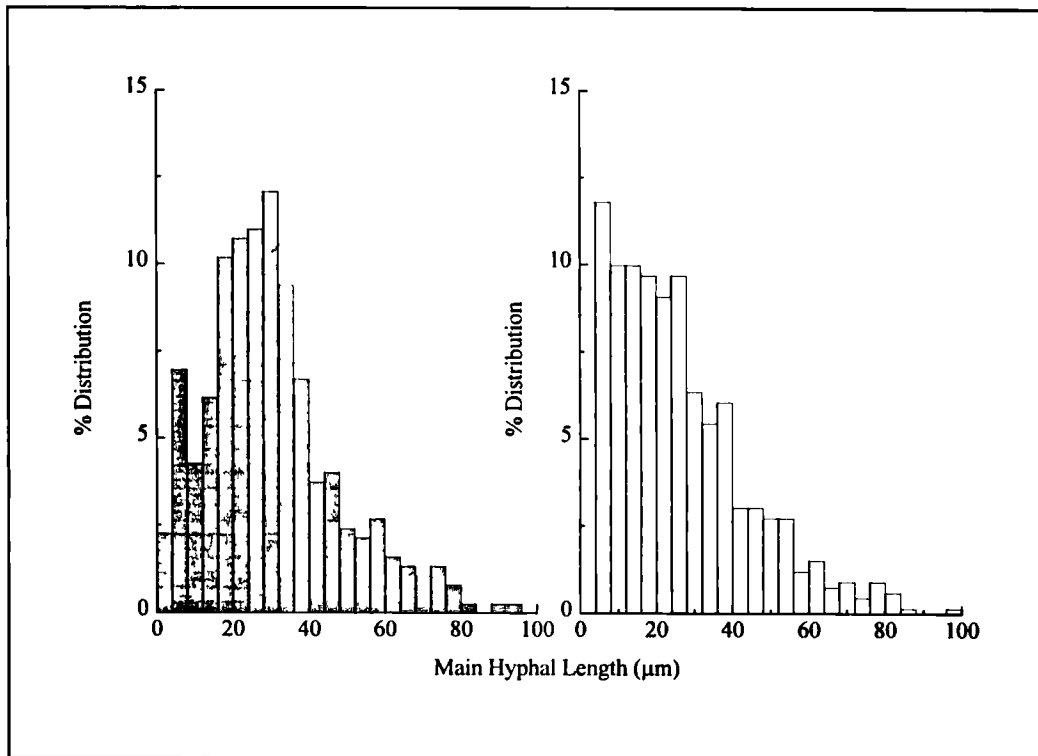
Belmar-Beiny and Thomas (1991) examined the effects of stirrer speed on morphology in *S. clavuligerus* (ATCC 20764) 5 L batch fermentations containing soluble complex media using image analysis. Morphology changed throughout the fermentation from clump to filamentous forms. Observations of hyphal regrowth during the later stages of fermentation were also reported. Conclusions were that growth showed little dependence on stirrer speed although morphological changes which occurred during each fermentation occurred earlier at the higher stirrer speeds.

Due to the differences noted in viscosity and capacitance of *S. clavuligerus* fermentations held at various tip speeds an investigation into the changes in hyphal morphology was carried out using image analysis. Morphological parameters compared are described in Table 3.8.. Characteristics of duplicate fermentations at tip speeds of  $2.83 \text{ m s}^{-1}$  and  $3.77 \text{ m s}^{-1}$  were analysed. Triplicate slides of each fermentation were analysed at 100 fields *per* slide.

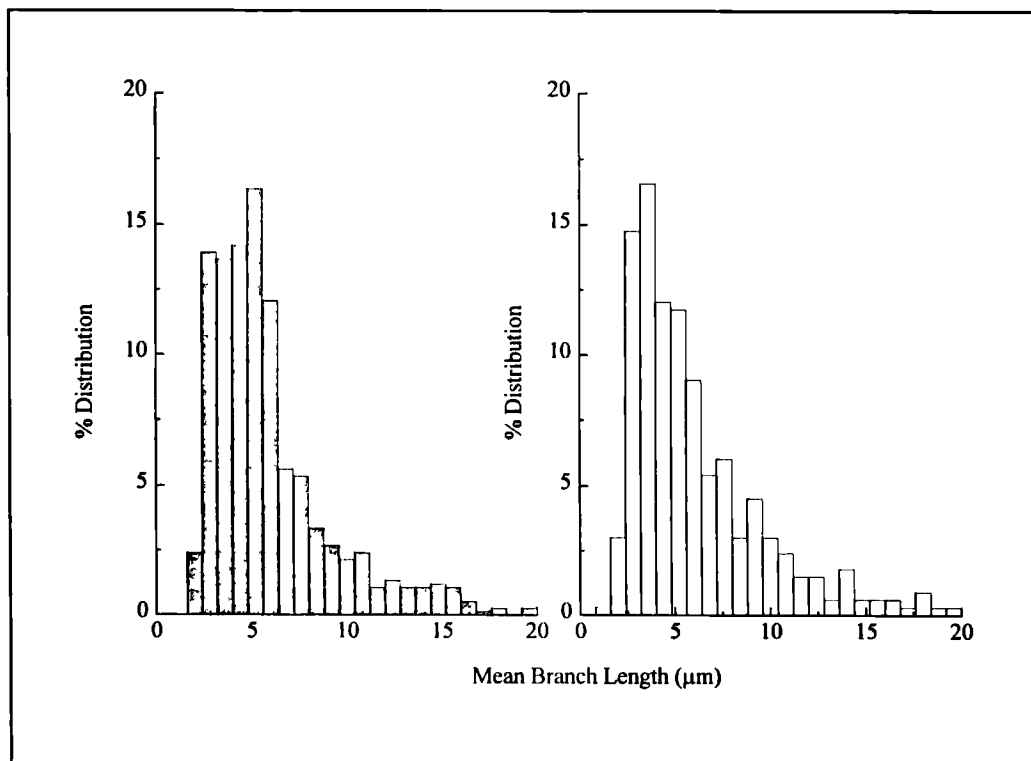
Belmar-Beiny and Thomas (1991) reported that morphological data was unreliable during initial stages of fermentation due to the high degree of hyphal entanglement. Although the industrial strain used in this study was more filamentous, initial microscopic observation revealed a high frequency of hyphal entanglement. A broad variation in broth viscosity and capacitance was observed at 72 h, Figure 5.2. and 5.3. Therefore samples taken at 72 h were analysed for morphological variations. Results were plotted as percentage distribution within a defined hyphal length for comparison of tip speed fermentations. Frequency distributions for the morphological parameters measured during image analysis of *S. clavuligerus* show a positive skew from normal distribution for all hyphal parameters measured, Figure 5.7.a.

Results show that on comparison of fermentations held at  $2.83 \text{ m s}^{-1}$  and  $3.77 \text{ m s}^{-1}$  there was no significant difference in the length of hyphal branches, Figure 5.7.b. and total hyphal length, Figure 5.7.c. Main hyphal lengths were smaller in the fermentation held at a tip speed of  $3.77 \text{ m s}^{-1}$  than at the lower tip speed. This is due to increased shear forces induced by the increase in tip speed accelerating hyphal fragmentation.

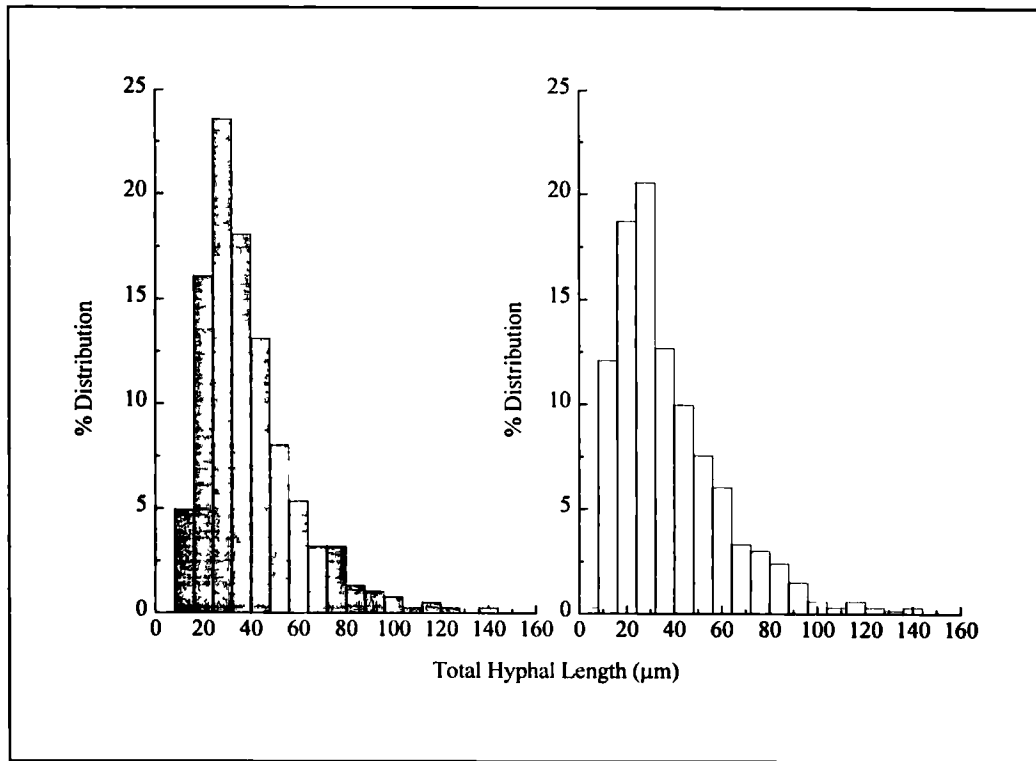
A significant variation was observed in the percentage distribution of filament area, Figure 5.7.d. Total filament area was lower in the fermentation held at the tip speed of  $3.77 \text{ m s}^{-1}$  when compared to the fermentation at the lower tip speed. This may be due to a decrease in thickness of hyphae cell walls and the hyphae themselves at the higher tip speed and may account for the decrease in broth viscosity and on-line change in capacitance at the fermentation held at  $3.77 \text{ m s}^{-1}$ .



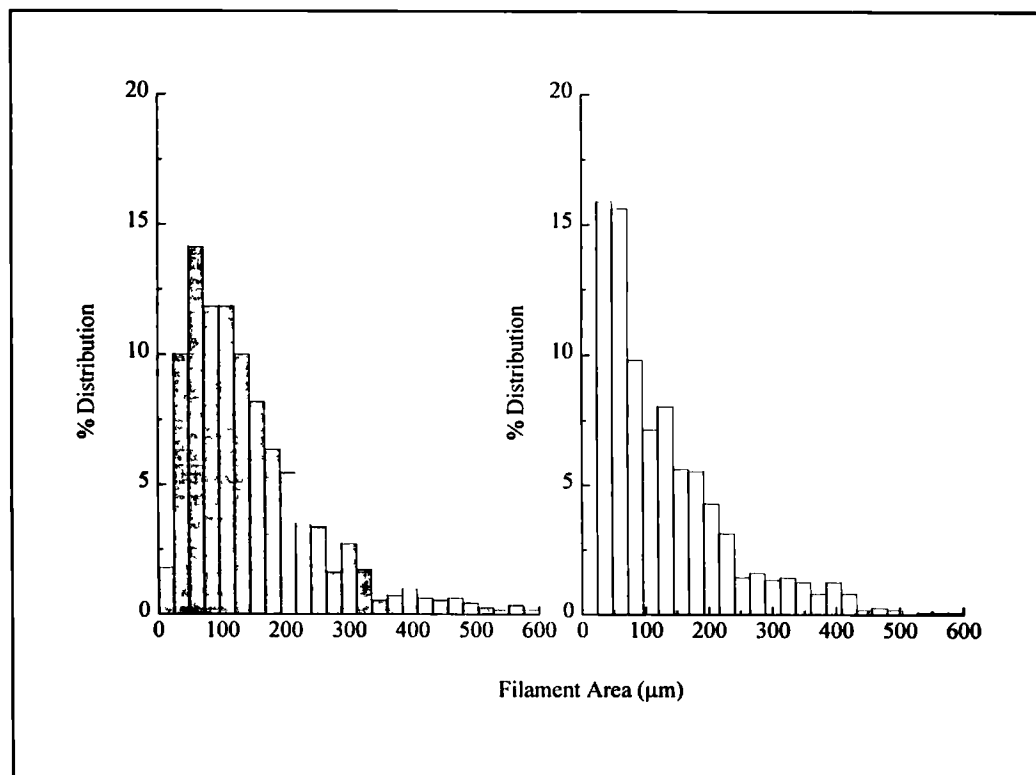
**Figure 5.7.a.** Percentage distribution of main hyphal length in fermentations held at tip speeds of 2.83 m s<sup>-1</sup> (■) and 3.77 m s<sup>-1</sup> (□).



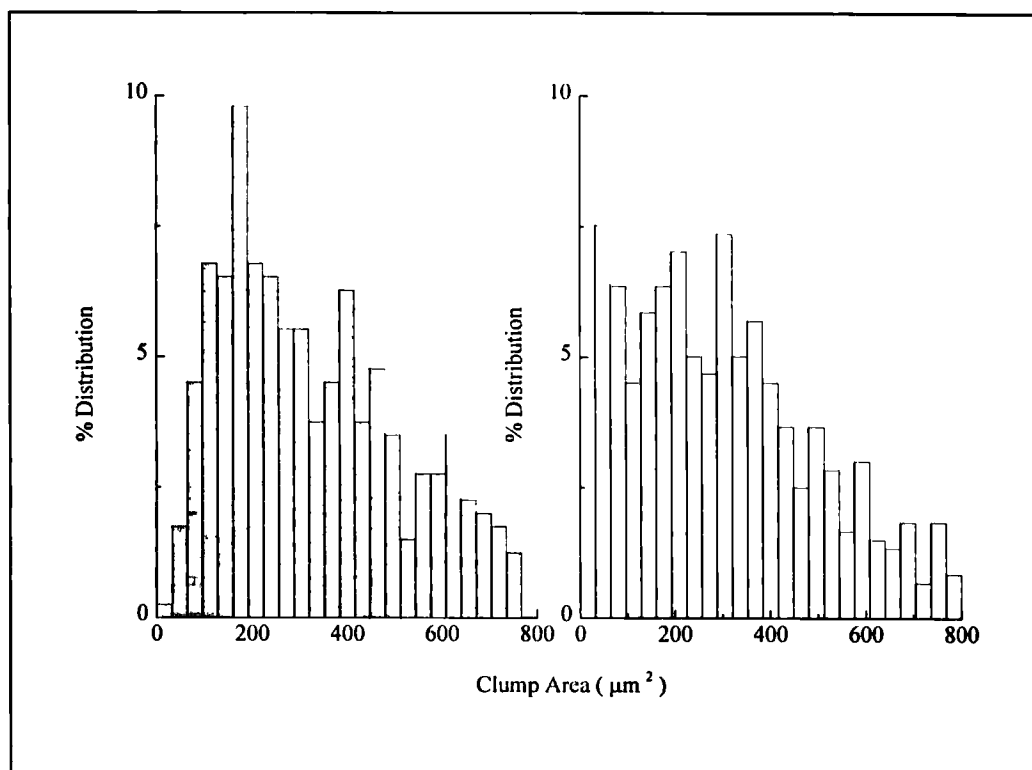
**Figure 5.7.b.** Percentage distribution of branch length in fermentations held at tip speeds of 2.83 m s<sup>-1</sup> (■) and 3.77 m s<sup>-1</sup> (□).



**Figure 5.7.c. Percentage distribution of total hyphal length in fermentations held at tip speeds of 2.83 m s<sup>-1</sup> (■) and 3.77 m s<sup>-1</sup> (□).**



**Figure 5.7.d. Percentage distribution of total filament area in fermentations held at tip speeds of 2.83 m s<sup>-1</sup> (■) and 3.77 m s<sup>-1</sup> (□).**



**Figure 5.7.e. Percentage distribution of clump area in fermentations held at tip speeds of  $2.83 \text{ m s}^{-1}$  (■) and  $3.77 \text{ m s}^{-1}$  (□).**

There may not be a significant difference in hyphal lengths on comparison of fermentations due to limitations within the image analysis data acquisition package. If a closed loop is formed in the filament the hyphae is regarded as a clump and not taken into account in hyphal length measurements. The probability of a loop occurring, in either the main hyphae or hyphal branches, is proportional to unit length. Hyphal morphology in *S. clavuligerus* is mainly filamentous and therefore clump area gave a better indication of hyphal dimensions due to the clump definition including a single closed loop. A comparison of clump areas are shown in Figure 5.7.e. The percentage distribution of clump areas at the higher tip speed was more widespread with a higher proportion of smaller clump areas.

## **5.2. The effect of tip speed on lipase activity**

An optimum physical environment within a bioreactor is essential for maximum lipase activity, Section 2.3.4. One of the most important factors to consider in a stirred tank is the effect of tip speed on enzyme activity, as many proteins are affected by shear forces and interfacial damage.



Results from the analysis of lipase activity, using titration against 0.01 M NaOH, were divided by viscosity as an indication of specific lipase activity, Figure 5.9. These data and that of previous workers (Sarra *et al.*, 1996) have shown that activity per unit viscosity is an acceptable means of comparison where specific activity cannot be calculated using dry weight or optical density. Capacitance was not used as the data was too noisy and an accurate figure at a specific point could not be calculated. Dissolved oxygen tension was maintained above 50% in all fermentations to eliminate changes in activity due to low oxygen tension. It can therefore be concluded that changes in lipase activity are due solely to the effects of changes in tip speed.

Volumetric lipase activity profiles, Figure 5.8, for constant tip speed fermentation show that there was a decrease in activity at the highest tip speed of  $3.77 \text{ m s}^{-1}$ . Results of constant tip speed fermentations shows maximum specific lipase activity at a tip speed of  $1.88 \text{ m s}^{-1}$ , Figure 5.9.a.. The level of specific lipase activity was reduced as tip speed was increased above  $2.36 \text{ m s}^{-1}$ . Figure 5.9.b. shows the effect of increasing tip speed during the fermentation. At the initial tip speed of  $2.83 \text{ m s}^{-1}$  the specific lipase increased. At 48 h the tip speed was increased and specific lipase activity remains constant. After 48h the specific activity follows a similar trend to that of the fermentation held at  $3.77 \text{ m s}^{-1}$ .

Cavanagh (1996) showed that lipase was not secreted from *S. clavuligerus* and may therefore be situated on, or associated with, the hyphal membrane. The reduction in specific lipase activity with increasing constant tip speed may be due to the effect of increased shear forces causing a dissociation of the enzyme from the cell membrane. Alternatively the configuration or the active site of the lipase protein itself may be affected by induced shear forces.

The profiles show that in the fermentation in which the tip speed was increased during the process the specific lipase activity did not increase after 48h. It was shown previously that hyphal fragmentation was reduced in this process due to the age of the hyphae and therefore specific activity was expected to increase further throughout this fermentation. The lipase protein may have encountered interfacial damage during intensive mixing by the increase in gas-liquid interfaces at the highest tip speed (Hoare *et al.*, 1993).

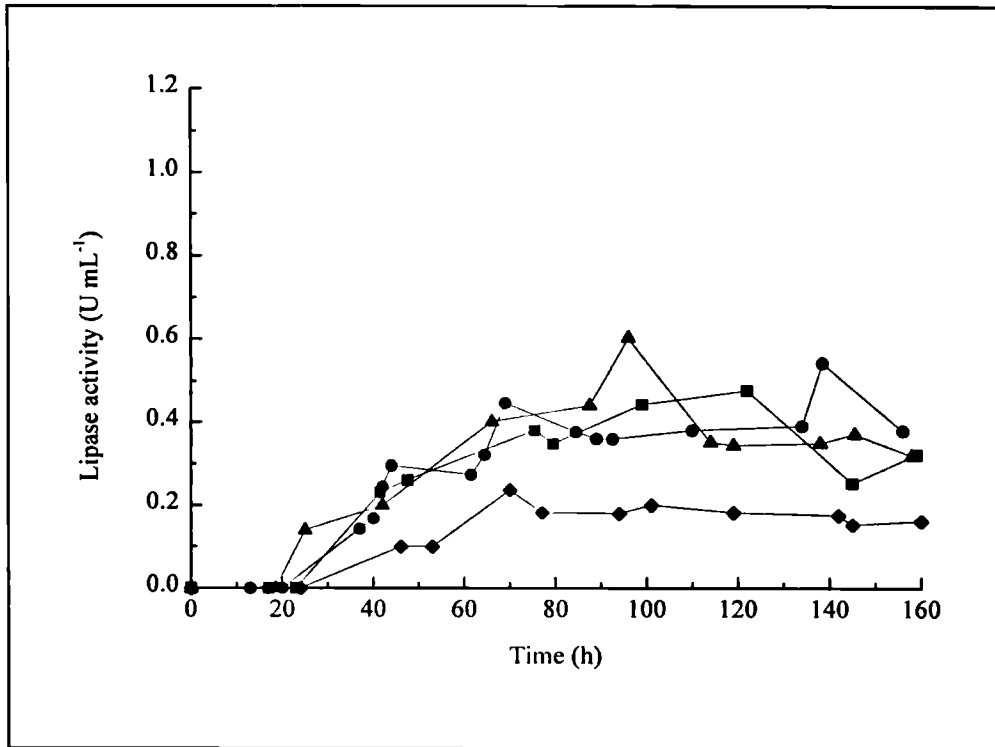


Figure 5.8.a. Volumetric lipase activity during batch cultivation at tip speeds of 1.88 m s<sup>-1</sup> (■), 2.36 m s<sup>-1</sup> (●), 2.83 m s<sup>-1</sup> (▲) and 3.77 m s<sup>-1</sup> (◆)

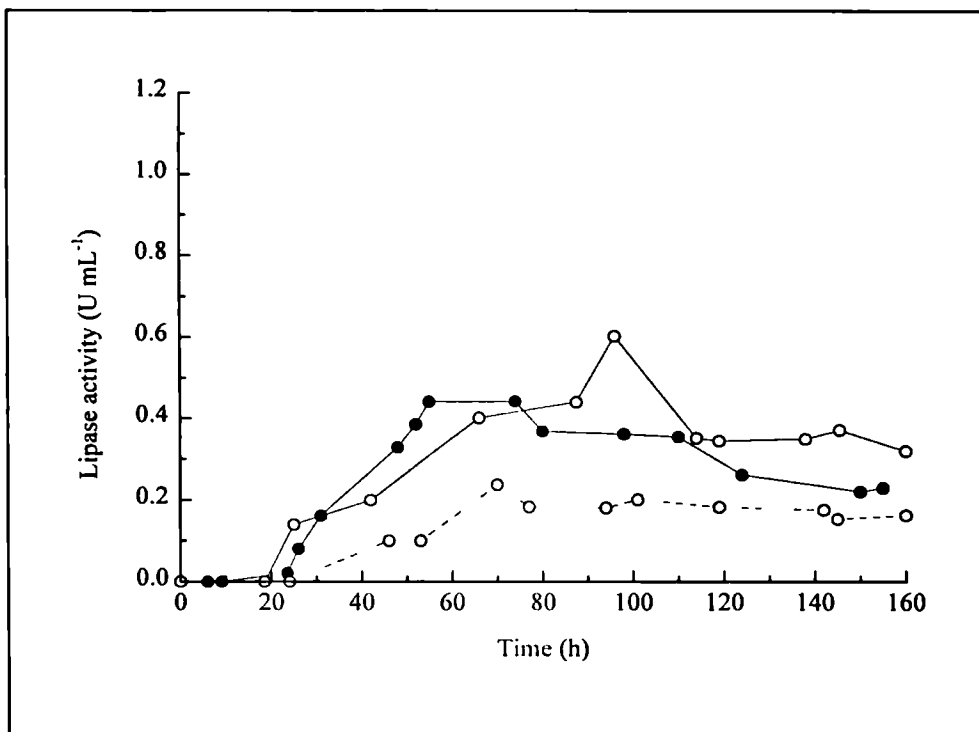
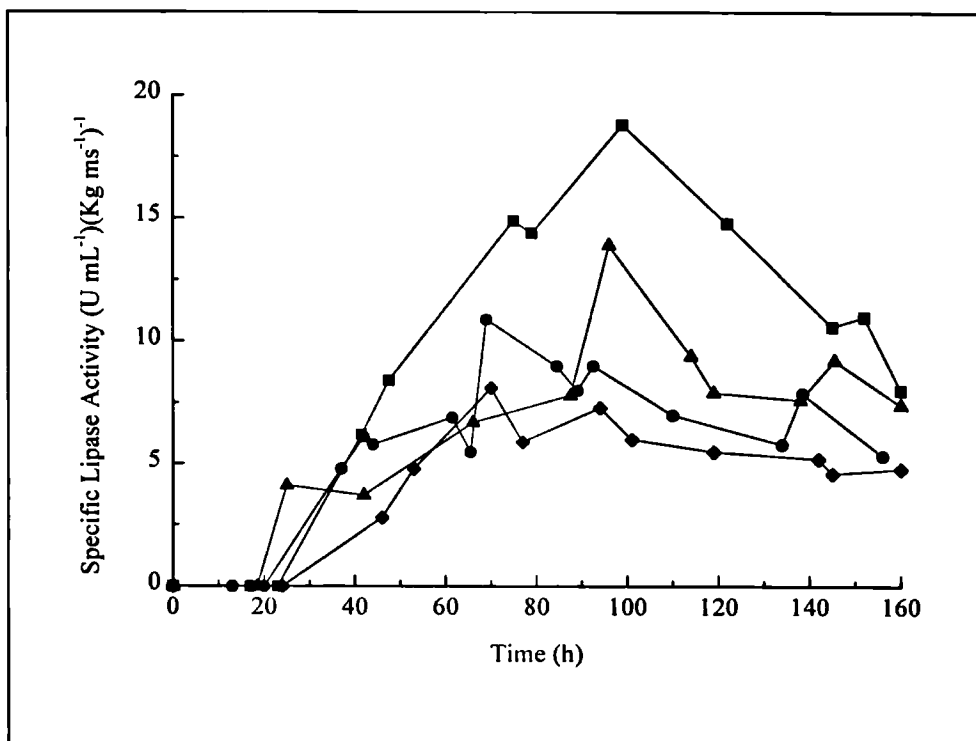
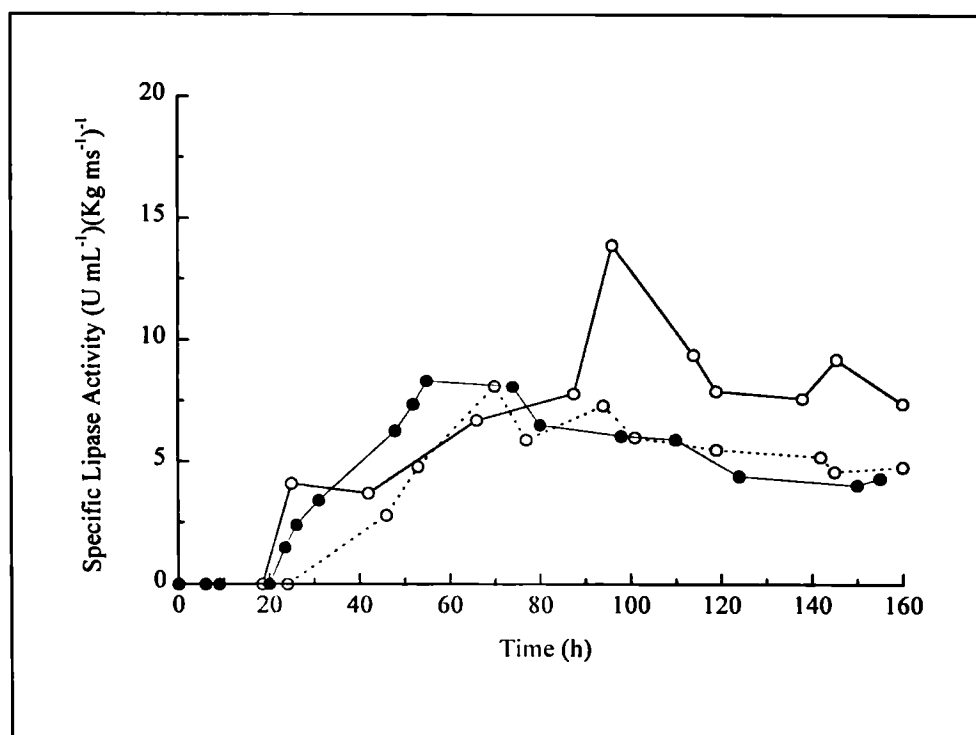


Figure 5.8.b. Actual lipase activity during batch cultivation at tip speeds of 2.83 m s<sup>-1</sup> (○), 3.77 m s<sup>-1</sup> (◌●) and 2.83 m s<sup>-1</sup> to 3.77 m s<sup>-1</sup> at 48 h (●)



**Figure 5.9.a.** Specific lipase activity during batch cultivation at tip speeds of  $1.88 \text{ m s}^{-1}$  (■),  $2.36 \text{ m s}^{-1}$  (●),  $2.83 \text{ m s}^{-1}$  (▲) and  $3.77 \text{ m s}^{-1}$  (◆)



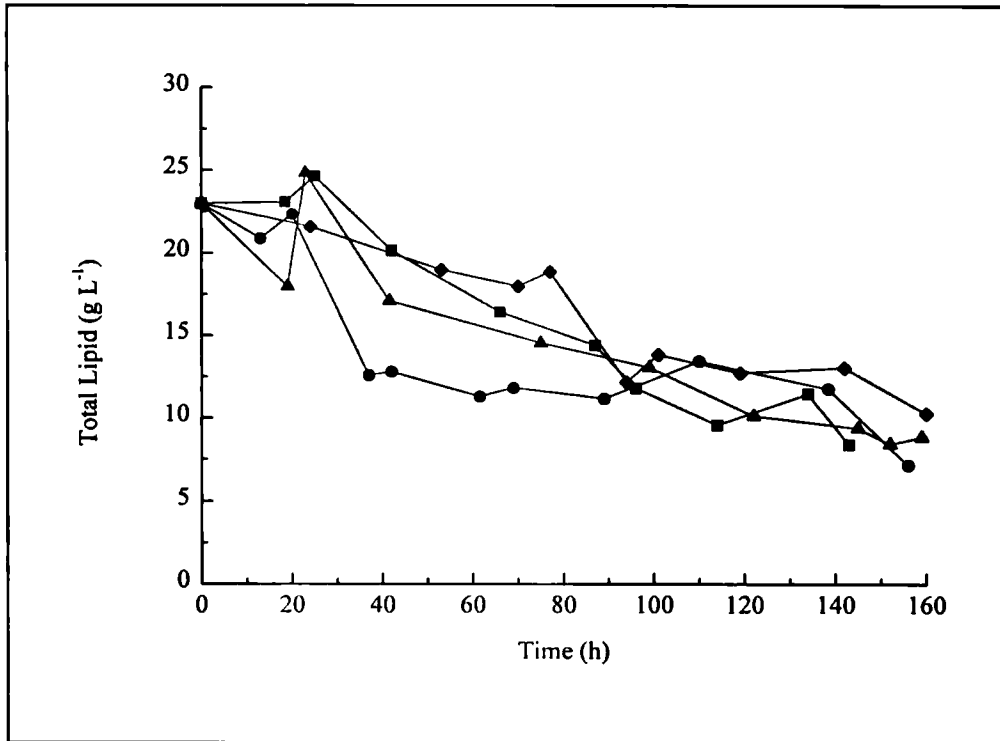
**Figure 5.9.b.** Specific lipase activity during batch cultivation at tip speeds of  $2.83 \text{ m s}^{-1}$  (○),  $3.77 \text{ m s}^{-1}$  (◌) and  $2.83 \text{ m s}^{-1}$  to  $3.77 \text{ m s}^{-1}$  at 48 h (●)

### **5.3. The effect of tip speed on lipid utilisation**

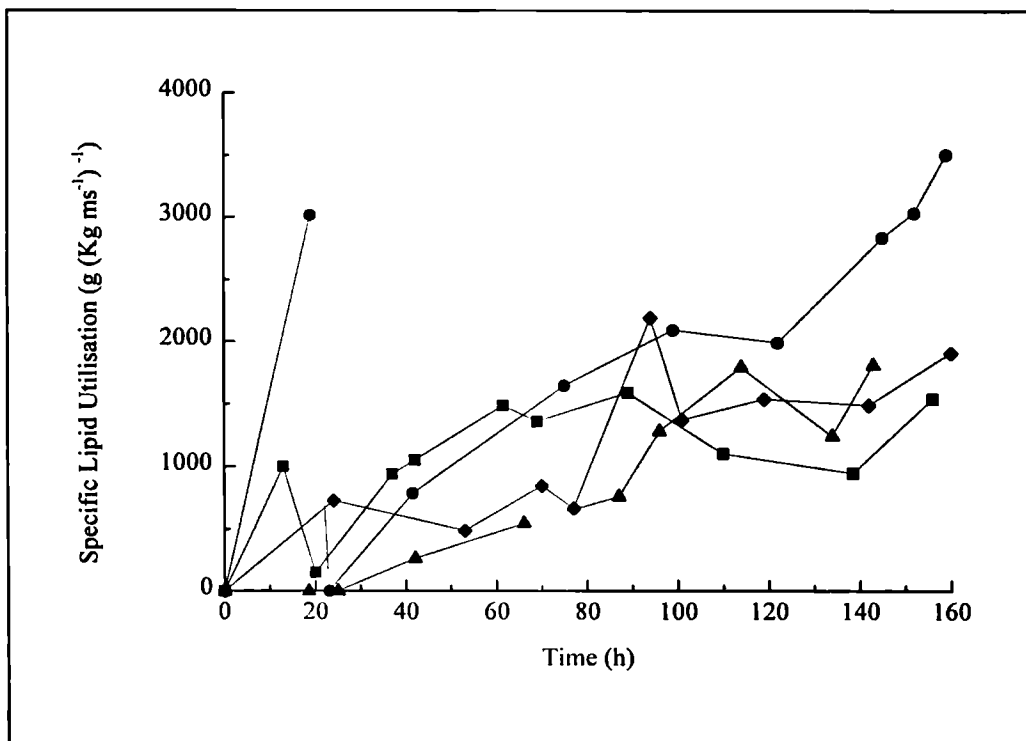
The aim of the tip speed study was to investigate the effects of impeller tip speed on rates of lipid utilisation and, more significantly, the residual lipid level. Increased oil utilisation will lead to an increase in clavulanic acid titres within the industrial fermentations of *S. clavuligerus* and reduce downstream processing operations.

Total lipid analysis for the tip speed comparison fermentations are shown in Figure 5.10. Standard deviations from the mean for the total lipid assay are not shown in error bars but ranged from approximately 15 % initially to 5 % towards the end of the fermentation. The results revealed that from all the fermentations carried out the increase in constant tip speed had no significant effect on the rapeseed oil used. To eliminate results due to the differences in cell concentration specific lipid utilisation was calculated for the constant tip speed fermentations using viscosity as a measure of cell concentration, Figure 5.11. Specific lipid utilisation was highest at  $2.36 \text{ m s}^{-1}$ .

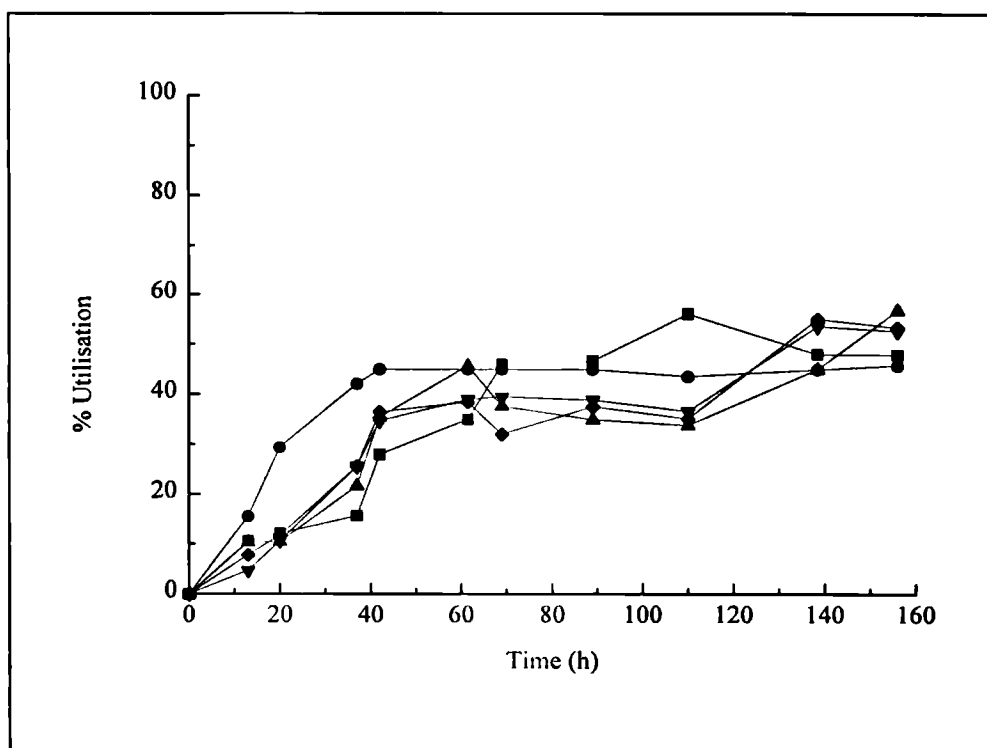
The most important result in terms of this study was that the residual oil level, approximately 30 % was the same regardless of the tip speed used. Residual oil levels were supported by analysis of fatty acid methyl esters using gas chromatography. All fatty acid utilisation profiles for the tip speed fermentations were similar, following the total lipid utilisation profiles, Figure 5.12. Results showed no particular specificity towards any of the fatty acid components of rapeseed oil. Specificity for stearic acid may be slight in the fermentations held at the lower tip speeds,  $1.88 \text{ m s}^{-1}$  and  $2.36 \text{ m s}^{-1}$ . Residual oil composition remained similar to the initial rapeseed oil present in the complex media.



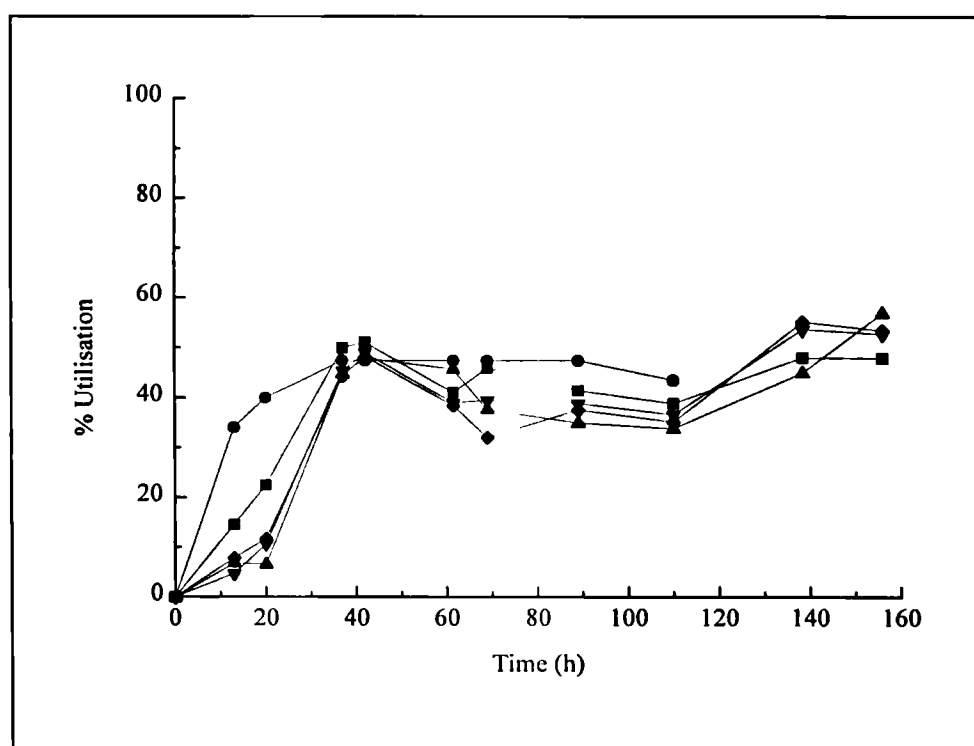
**Figure 5.10.** Residual oil levels in batch cultivation at tip speeds of  $1.88 \text{ m s}^{-1}$  (■),  $2.36 \text{ m s}^{-1}$  (●),  $2.83 \text{ m s}^{-1}$  (▲) and  $3.77 \text{ m s}^{-1}$  (◆)



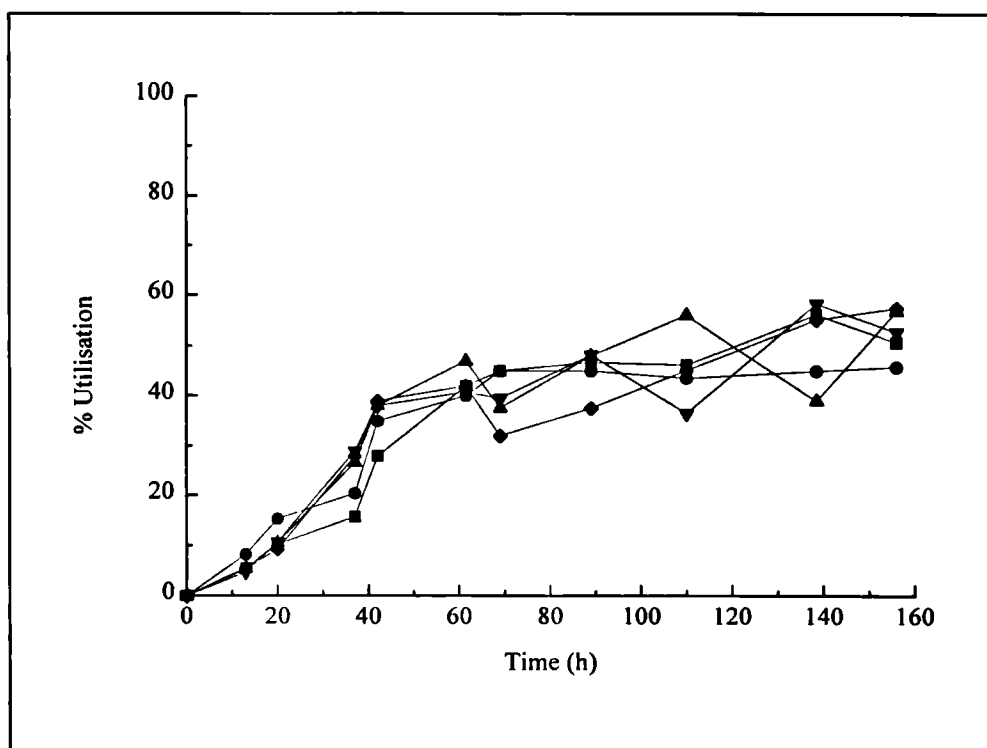
**Figure 5.11.** Specific lipid utilisation in fermentation at tip speeds of  $1.88 \text{ ms}^{-1}$  (■),  $2.36 \text{ ms}^{-1}$  (●),  $2.83 \text{ ms}^{-1}$  (▲) and  $3.77 \text{ ms}^{-1}$  (◆)



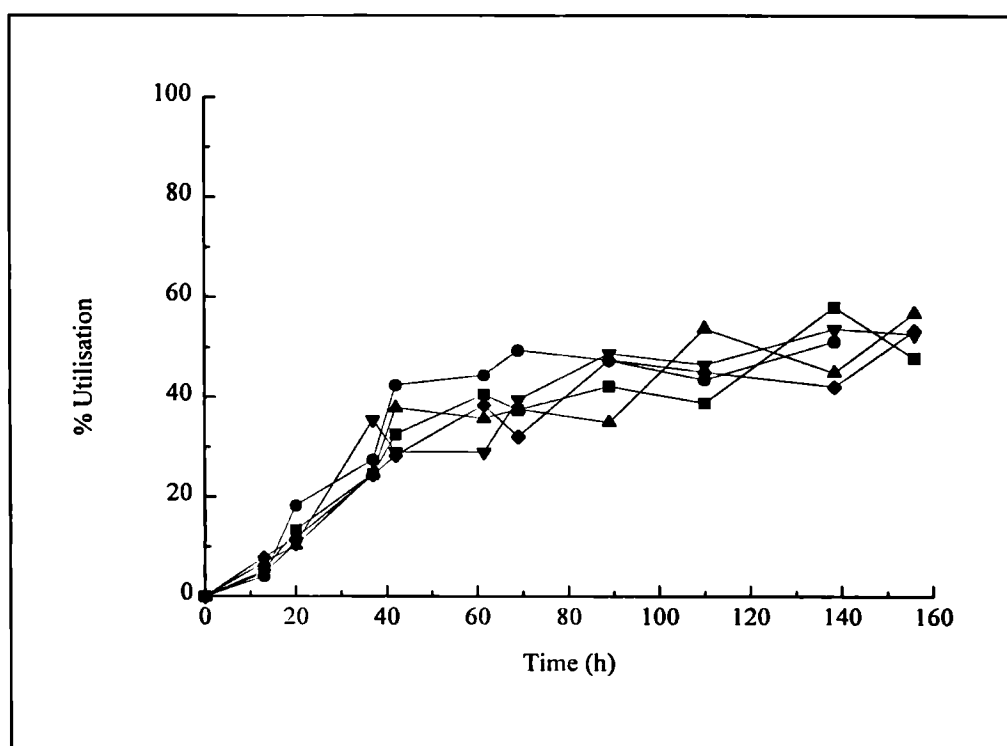
**Figure 5.12.a.** Fatty acid utilisation in a fermentation at a tip speed of  $1.88 \text{ m s}^{-1}$   
*Palmitic (■), Stearic (●), Oleic (▲), Linoleic (▼) and Linolenic acid (◆).*



**Figure 5.12.b.** Fatty acid utilisation in a fermentation at a tip speed of  $2.36 \text{ m s}^{-1}$   
*Palmitic (■), Stearic (●), Oleic (▲), Linoleic (▼) and Linolenic acid (◆).*



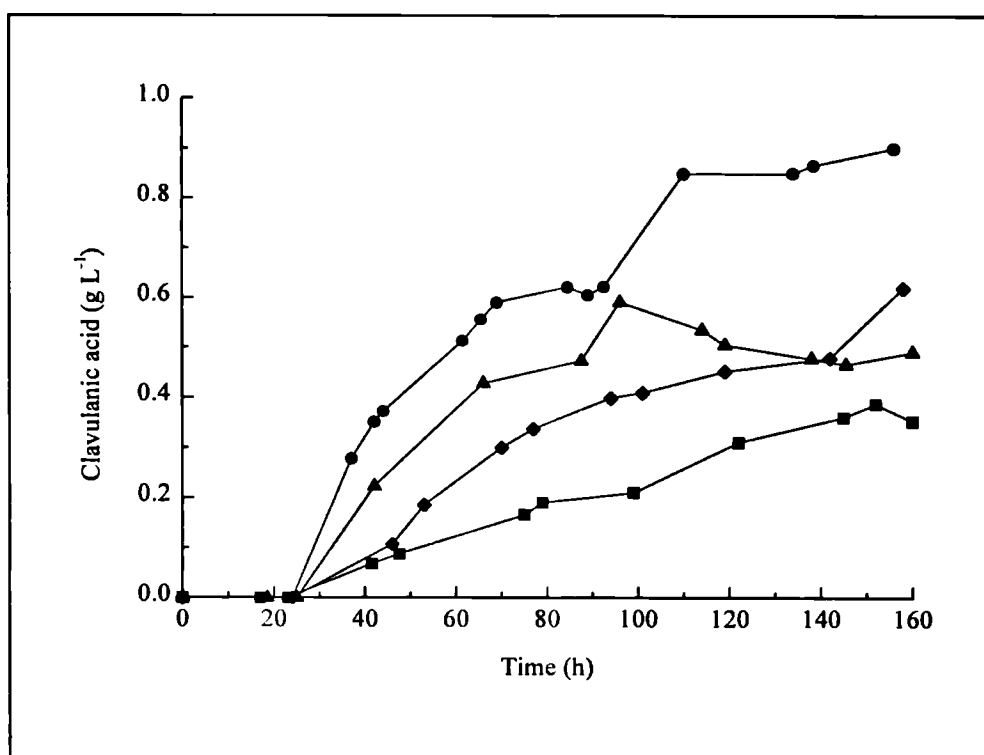
**Figure 5.12.c.** Fatty acid utilisation in a fermentation at a tip speed of  $2.83 \text{ m s}^{-1}$   
*Palmitic (■), Stearic (●), Oleic (▲), Linoleic (▼) and Linolenic acid (◆).*



**Figure 5.12.d.** Fatty acid utilisation in a fermentation at a tip speed of  $3.77 \text{ m s}^{-1}$   
*Palmitic (■), Stearic (●), Oleic (▲), Linoleic (▼) and Linolenic acid (◆).*

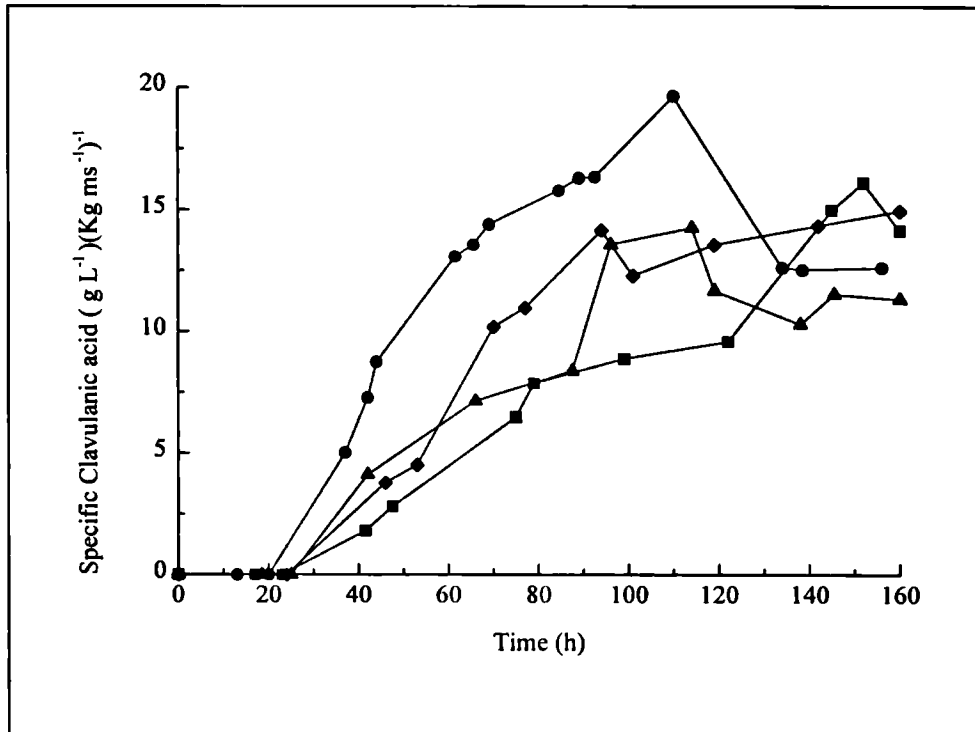
#### 5.4. The effect of tip speed on productivity

Published results in the study of the effects of stirrer speed on the production of clavulanic acid in submerged cultures of *S. clavuligerus* have shown that productivity may be both affected and independent on tip speed, (Belmar-Beiny and Thomas, 1991; Tarbuck *et al.*, 1985). The results for volumetric and specific clavulanic acid production carried out in the constant tip speed study are shown in Figure 5.13.a. and b respectively. Although actual titres varied considerably, specific production was not significantly dependent on stirrer speed although higher productivity was seen at  $2.36 \text{ m s}^{-1}$  indicating an optimal stirrer speed for maximum productivity.

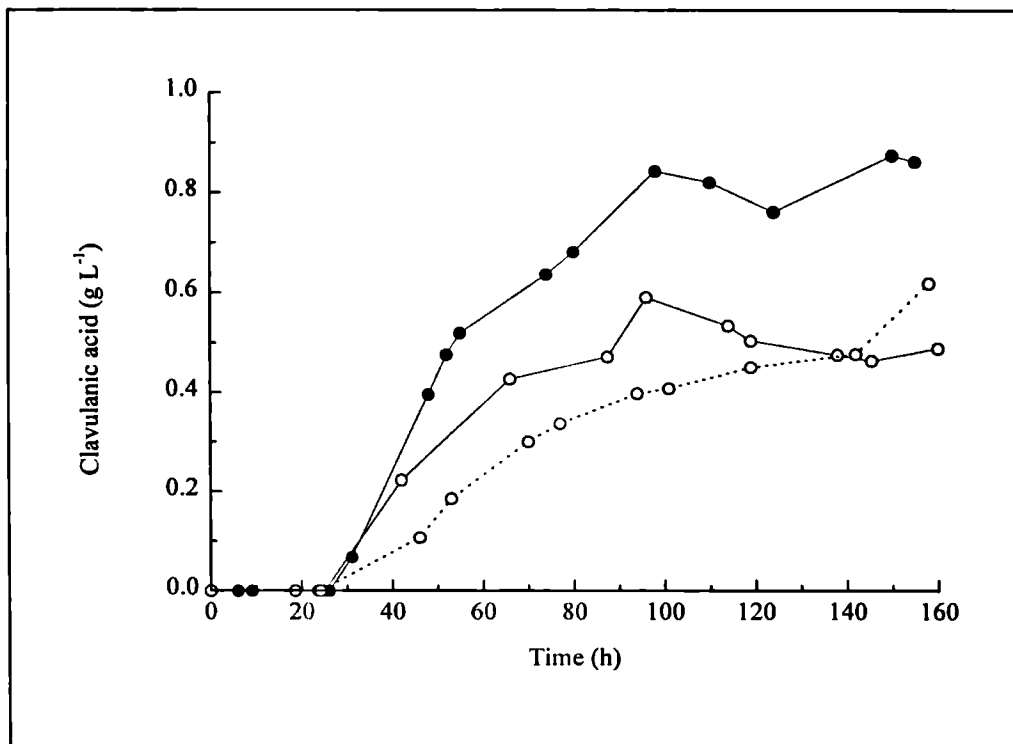


**Figure 5.13.a.** Volumetric clavulanic acid production at tip speeds of  $1.88 \text{ m s}^{-1}$  (■),  $2.36 \text{ m s}^{-1}$  (●),  $2.83 \text{ m s}^{-1}$  (▲) and  $3.77 \text{ m s}^{-1}$  (◆)

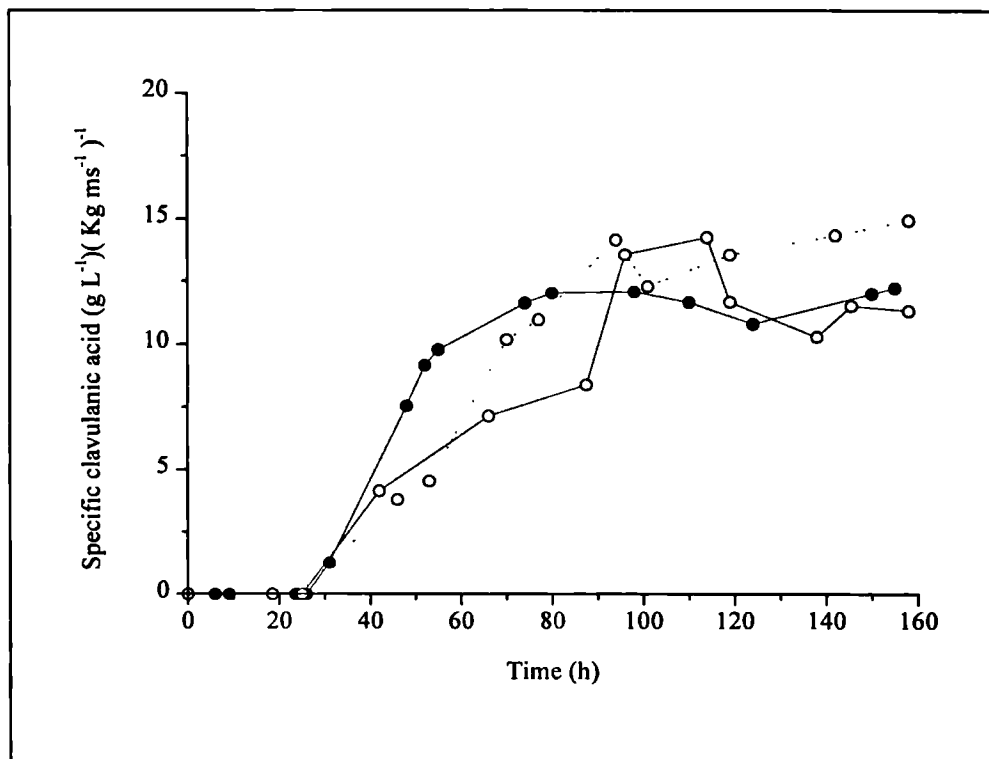




**Figure 5.13.b.** Specific clavulanic acid production at tip speeds of  $1.88 \text{ m s}^{-1}$  (■),  $2.36 \text{ m s}^{-1}$  (●),  $2.83 \text{ m s}^{-1}$  (▲) and  $3.77 \text{ m s}^{-1}$  (◆)



**Figure 5.13.c.** Volumetric clavulanic acid production during fermentation at tip speeds of  $2.83 \text{ m s}^{-1}$  (○),  $3.77 \text{ m s}^{-1}$  (◌) and  $2.83 \text{ m s}^{-1}$  to  $3.77 \text{ m s}^{-1}$  at 48 h (●)



**Figure 5.13.d. Specific clavulanic acid production during fermentation at tip speeds of  $2.83 \text{ m s}^{-1}$  (○),  $3.77 \text{ m s}^{-1}$  (◐) and  $2.83 \text{ m s}^{-1}$  to  $3.77 \text{ m s}^{-1}$  at 48 h (●)**

Figures 5.13.c. and d. shows that with an increase in tip speed at 48 h productivity is unaffected. As oil utilisation was unaffected by tip speed it was expected that specific productivity would also remain the same.

## 5.5. Discussion

It is often difficult in batch cultivation of filamentous microorganisms to distinguish the effects due to changes in stirrer speed from consequential changes in the dissolved oxygen tension levels. In order to eliminate the effects due to changes in oxygen transfer levels in this study the dissolved oxygen level was held above 50%.

Maximum biomass achieved was found to increase when tip speed was increased from  $1.88 \text{ m s}^{-1}$  to  $2.83 \text{ m s}^{-1}$ . A tip speed of  $3.77 \text{ m s}^{-1}$  was detrimental to growth. These data indicate an optimal stirrer speed for growth defined as a balance between the effect of increased mass transfer and shear forces induced. Effects of stirrer speed on the growth of *S. clavuligerus*, using dry weight analysis (Belmar-Beiny and Thomas, 1991) showed that cell growth was not particularly shear sensitive whereas Bronnenmeir and

Markl (1982) showed that growth rates of several fungi are sensitive to changes in stirrer speed. Changes in viscosity during the process shown in this study indicate that increasing the tip speed from  $1.88 \text{ m s}^{-1}$  to  $2.83 \text{ m s}^{-1}$  increased biomass concentration but an increase to  $3.77 \text{ m s}^{-1}$  on a 5 L batch scale accelerates hyphal fragmentation. These results are supported by photographic evidence of the differences in morphology and image analysis of hyphal lengths.

An advantage of using the on-line capacitance base biomass probe over the traditional dry weight method of measuring changes in biomass concentration is that since only biomass with intact cellular membranes contribute to the capacitance measurement, the probe does not detect cell debris. Therefore as the biomass probe results indicate, the increase in bioreactor tip speed to  $3.77 \text{ m s}^{-1}$  was detrimental to growth due to an increase in hyphal fragmentation. On-line biomass monitor data highlight the disadvantages of using off-line viscosity measurements where rapid changes between samples may not be detected.

Exit gas data revealed that although at a tip speed of  $3.77 \text{ m s}^{-1}$  viscosity and capacitance were lowest, oxygen uptake rates and carbon dioxide evolution rates were similar to those at the lower tip speeds. These data indicate that the viscosity and capacitance data were susceptible to changes in organism morphology and not representative of cell activity.

On comparison of tip speed fermentations image analysis data concluded the decrease in main hyphal length with an increase in tip speed from  $2.83 \text{ m s}^{-1}$  to  $3.77 \text{ m s}^{-1}$ . No significant difference was observed in hyphal branch lengths and total hyphal length. It is difficult to achieve reproducible batch fermentations of *Streptomyces*, particularly with respect to morphological measurements.

Results show maximum lipase activity at  $1.88 \text{ m s}^{-1}$  and a reduction in activity as tip speed is increased above  $2.36 \text{ m s}^{-1}$ . As dissolved oxygen tension was maintained above 50% it can be concluded that the reduction in lipase activity is due to the effects of changes in tip speed. Kinetics and mechanism of shear inactivation of a lipase from *Candida cylindracea* have been investigated by Lee and Choo (1989), concluding a loss in enzyme activity as a function of shearing times and shear rates. The lipase enzyme in

*S. clavuligerus* has been found not to be secreted and may be cell membrane associated (Cavanagh, 1996). As high tip speeds were responsible for increased fragmentation of the hyphae, increasing shear forces may affect the link between the enzyme and the cellular membrane hence decreasing activity or reducing the ability of the organism to synthesise the enzyme. Alternatively, the configuration or active site of the lipase protein itself may be affected by the shear forces induced.

The lipase enzyme may also encounter interfacial damage during intensive mixing by the increase in gas-liquid interfaces (Hoare *et al.*, 1993). Using small scale experiments as mimics of industrial equipment researchers demonstrated that proteins, such as lipase, can be affected by interfacial damage at a gas liquid interface, the rate of which can be enhanced by intensive mixing. Loss of activity may be avoided even in intensive mixing environments provided care is taken in design of process equipment to avoid interfacial damage.

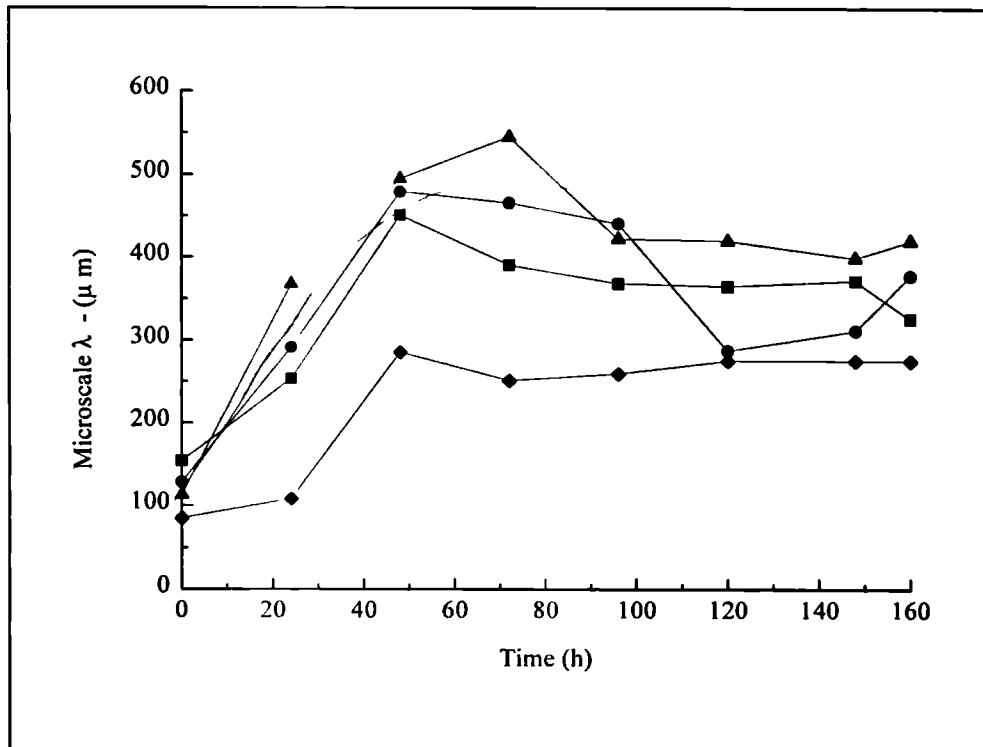
Lipase activity has also been shown to be affected by the size of the interfacial area of oil droplets. Extracellular lipase production was found to increase in *Candida rugosa* with increasing drop size of the dispersed phase, oleic acid i.e. at lower tip speeds (Dalmau *et al.*, 1998). The results of this work showed that oleic acid drop size was the most influential process parameter on comparison of different bioreactors in order to achieve similar lipase production.

Although specific lipase activity varied according to the increase in stirrer speed, lipid utilisation remained independent of tip speed. According to Hinze (1955) drop breakage occurs when the fluid dynamic forces tending to deform a droplet exceed the interfacial tension forces resisting the deformation. Therefore increasing tip speed may increase the forces tending to deform the oil droplet hence reducing droplet size. It was thought that the oil remaining at the end of the process may be due to a physical limitation on mass transfer therefore increasing the surface area to volume ratio may increase the accessibility of the oil to the organism increasing metabolism and decreasing the residual oil level. As lipases are reported to interact at the oil/water interface (Macrae, 1989) increasing the tip speed was also intended to decrease droplet size in order to increase the interfacial area available to the lipase.

Lipid utilisation was unaffected by tip speed as the increase did not decrease the size of the rapeseed oil droplets due to the microscale of turbulence being larger than the size of largest oil droplets. The microscale of turbulence throughout the process at each tip speed was determined using the Kolmogorov microscale (Kolmogorov, 1941). The degree of homogeneity possible in a bioreactor as a result of mixing is limited by the size of the smallest eddies. Dispersion of the process media i.e. lipid droplets, into smaller and smaller eddies will facilitate rapid transfer of material throughout the bioreactor and may increase oil utilisation. The degree of homogeneity possible as a result of dispersion is limited by the size of the smallest eddies which may be formed in the process media. The size of the smallest eddy is given by the Kolmogorov scale of micro turbulence ( $\lambda$ ). A sample calculation is given in Appendix 1.

The microscale of turbulence for the fermentations held at different agitation rates is shown in Figure 5.14. Particle size analysis of rapeseed oil droplets revealed the size of the largest oil droplets, at relevant tip speeds, to be approximately 100-150  $\mu\text{m}$  (Cavanagh *et al.*, 1994). Figure 5.14. shows that, initially, the scale of mixing will reduce the size of the oil droplets but, due to the changes in process viscosity, at a given tip speed the microscale of turbulence increases. Increasing the tip speed, although reducing media viscosity, will not reduce the viscosity enough to decrease the size of the average oil droplet. Ramping tip speed during fermentation did not reduce viscosity throughout the process and therefore did not decrease microscale of turbulence.

The equation for the microscale of turbulence, Appendix 1, shows the size of the smallest eddy below which mixing takes place by diffusion is created from power input to the bioreactor and viscosity of the process media. One way to decrease the microscale would be to increase the bioreactor power input. In this study on a 5 L scale the upper limit of tip speed was used and yet did not decrease the microscale to a level of oil droplet breakage. On industrial scale-up such a high power input would not be economically viable.



**Figure 5.14.** Microscale of turbulence throughout the process at tip speeds of  $1.88 \text{ m s}^{-1}$  (■),  $2.36 \text{ m s}^{-1}$  (●),  $2.83 \text{ m s}^{-1}$  (▲) and  $3.77 \text{ m s}^{-1}$  (◆).

The impeller used during the tip speed fermentations was a Rushton turbine with a diameter of 0.06 m and a power number  $3 \times 4.5$ . As impeller diameter and power number contribute to power input the microscale of turbulence could be reduced by increasing both parameters. The power number of a Rushton turbine may be increased by increasing the size of the blades or the number of impellers used. The effect of increasing the diameter of the impeller from 0.06 m to 0.07 m on the 5 L scale used is shown in Table 5.2.

**Table 5.2.** Microscale of turbulence dependent on impeller diameter

Impeller diameter (m)	0.06	0.07
Pug (W)	10.50	22.69
Pg (W)	4.03	9.92
Pg/V (W m <sup>3</sup> )	805.10	1938.84
λ (μm)	143	115

Another way to decrease the microscale of turbulence is to decrease the media viscosity. This may have a more significant effect as this component of the equation is to the power three. Reducing media viscosity may be accomplished by addition of components such as surfactants or by media dilution. A significant dilution is required to reduce the media viscosity within the bioreactor to a level at which a decrease in microscale of turbulence to decrease drop size. A dilution of 1:1 would only reduce the viscosity by 35%. Dilution is not feasible on industrial scale-up due to limitation and value of bioreactor space and will not reduce the microscale of turbulence sufficiently to reduce oil droplet size.

Homogenisation of the media pre-sterilisation and/or addition of a surfactant to the media may reduce or stabilise oil droplet size. Once the droplet size is decreased oil utilisation may increase, hence increasing overall product titres. An increase in product titre due to increased oil utilisation must be weighed up against the effect of additional processing and surfactant addition on cell growth and productivity and the cost of their downstream removal.

The effect of stirrer speed on clavulanic acid production in *S. clavuligerus* has previously been investigated by Belmar-Beiny and Thomas (1991). Clavulanic acid production showed little dependence on stirrer speed and no link between the morphological changes and clavulanic acid production. Observations by Tarbuck *et al.* (1985) of clavulanic acid production by *S. clavuligerus* in 10 L batch fermentations suggest a drop in production with an increase in stirrer speeds. Results from the clavulanic acid production and increasing tip speeds in this study showed that production is relatively independent of stirrer speed. Image analysis results from tip speed fermentations conclude that productivity is also relatively independent of changes in hyphal morphology. The data in this study indicates that having strong cells does not affect production or the secretion of clavulanic acid in 5 L batch fermentation of *S. clavuligerus*.

Results presented in this chapter have highlighted the value of the on-line biomass probe for monitoring growth patterns in cultures where the medium contains oils and other insoluble components. It is also valuable in reflecting changes in culture morphology which in this case correlate well with viscosity. Research has also shown that lipase

activity decreases with increased tip speed which would be expected given that the lipase may be associated with the hyphal membrane (Cavanagh, 1996) and that the hyphae or clumps were smaller than the microscale of turbulence (Belmar-Beiny and Thomas, 1991). The rate of lipid utilisation and residual oil levels were more consistent with the microscale of turbulence but since both were dependent of the lipase activity further investigation into the physical environment within the process is required in order to understand the limitation on oil utilisation.



## **5.6. Summary of the effects of agitation rate on lipid utilisation**

Increasing tip speed extended hyphal development in *S. clavuligerus* to a point above which fragmentation occurred. Ramping tip speed during fermentation reduced hyphal fragmentation. Fragmented hyphae showed similar respiratory data to less fragmented mycelia.

Increasing tip speed from  $1.88 \text{ m s}^{-1}$  (600 rpm) to  $3.77 \text{ m s}^{-1}$  (1200 rpm) decreased specific lipase activity. This was attributed to the increase in shear forces affecting the membrane interaction of the lipase enzyme, the conformation of the enzyme itself, or interfacial inactivation.

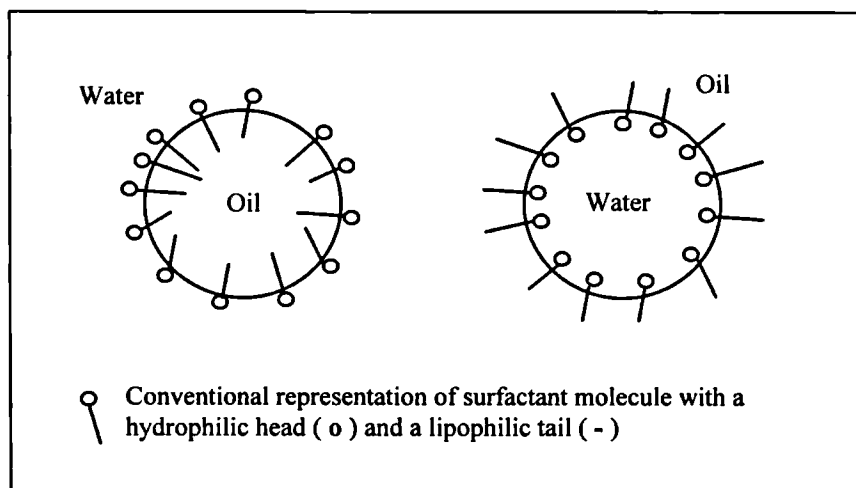
Increasing tip speed had no significant effect on lipid utilisation rates. Residual oil levels remained high, at approximately 30%, regardless of tip speed used. Gas chromatography analysis revealed no specificity in terms of fatty acid utilisation in all tip speed fermentations. The composition of the residual oil was similar to initial rapeseed oil.

Experiments showed that  $2.36 \text{ m s}^{-1}$  (750 rpm) was the optimal tip speed for clavulanic acid production in 5 L batch fermentations of *S. clavuligerus* using complex process media containing rapeseed oil.

Increasing tip speed did not decrease the microscale of turbulence, in a 5 L batch process, to a level at which drop breakage will occur. This was attributed to the viscosity of the complex media and limitations of power input with respect to hyphal damage. Residual oil levels in the complex media cannot be decreased by increasing tip speed.

## **6. THE EFFECT OF SURFACTANT ADDITION ON LIPID UTILISATION IN *S. CLAVULIGERUS***

In a fermentation using hydrocarbon as a substrate, the hydrocarbon may be dispersed in an aqueous system as fine droplets on addition of a surfactant. Surfactants are an interesting class of materials due to their hydrophilic and lipophilic nature. This has led to their use in many industries, including use in washing cloth, microelectronics, industrial separations, environmental protection and in enhancing oil recovery (Wasan *et al.*, 1988). The ratio of hydrophilic and lipophilic entities gives the surfactants their different properties. All surfactants, for example, emulsifiers, wetting agents, detergents and defoamers, lower the surface tension of water. Micelles may be formed when the concentration of surfactant reaches the critical micelle concentration, that is when the surface of the liquid is saturated with surfactant molecules. In an oil-water system the micelles form a microemulsion. Two types of microemulsion exist: water-in-oil and oil-in-water, Figure 6.1. Micro emulsions offer several interesting features as media for enzymic reactions and several research groups have investigated enzymic hydrolysis of triacylglycerides in microemulsions, usually of the water in oil type (Skagerlind and Holmberg, 1994).



**Figure 6.1. Schematic diagram for oil-in-water and water-in-oil microemulsion structures.**

As tip speed experiments did not increase lipid utilisation the effects of addition of a detergent, sodium dodecyl sulphate (SDS), to the complex media was investigated. Addition of detergent to the medium will lower surface tension of water therefore

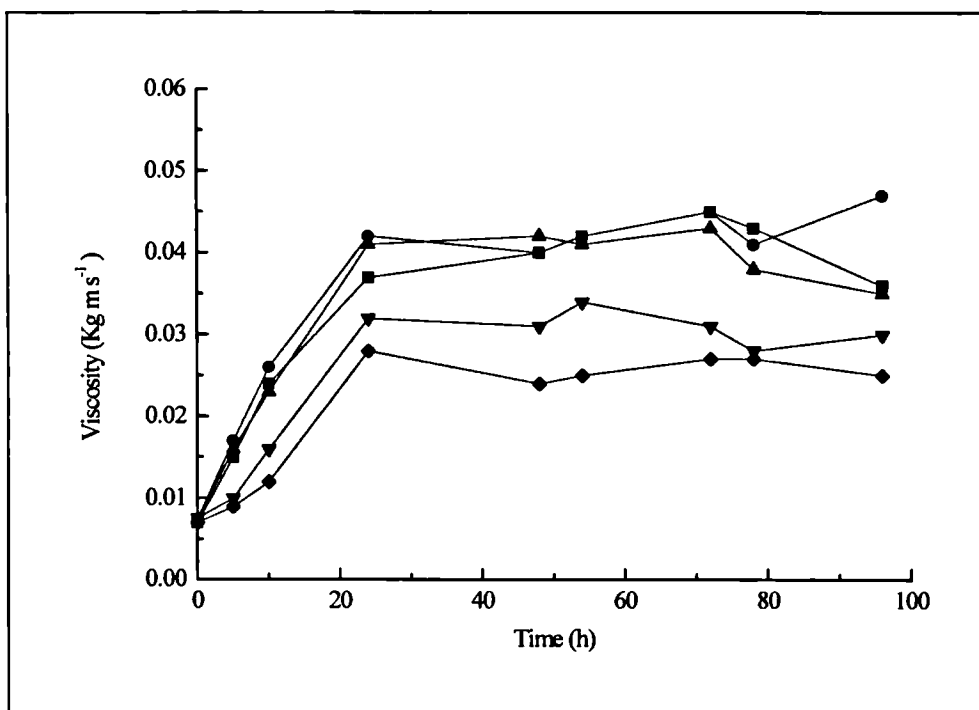
lowering the air-liquid barrier to oxygen transfer. The second detergent action of SDS is related to their foaming properties. The formation of fine bubbles is favourable to oxygen transfer as the total gas-liquid interfacial area increases. This action is counteracted by the necessary addition of more antifoam or a defoamer. SDS also has a low emulsifying power on the oil which may increase lipase activity and hence rapeseed oil utilisation.

5 L batch fermentations were carried out in a 7 L LH series 2000 vessel, Figure 3.1. DOT was maintained above a limiting level by mixing oxygen with the inlet air where necessary. Air flow rate was kept constant at 1 vvm. Duplicates of each fermentation was carried out and average results plotted.

### **6.1. Medium preparation**

Initial shake flask experiments were carried out to determine the level of SDS to be used in the comparative fermentations, Figure 6.2. 0.3% and 0.5% SDS had an adverse effect on viscosity levels. This may be due to the SDS lowering the viscosity of the medium or could be related to a decrease in growth. Levels below 0.1% SDS were investigated in the 5 L batch fermentations as these levels had been shown to have no adverse effect on growth. SDS was added to the media before *in situ* steam sterilisation at concentrations of 0.05%, 0.075% and 0.1%.

Additional antifoam was needed in all fermentations containing SDS due to its detergent action and increased dispersion of the lipid inhibiting the oils natural antifoam properties. The antifoam used was a lipid based industrial antifoam supplied by SmithKline Beecham Pharmaceuticals used in the manufacturing process. A lipid based antifoam will interfere with total lipid analysis and therefore control fermentations with additional antifoam were carried out to limit the difference in total lipid analysis and results due to changes in oxygen transfer.

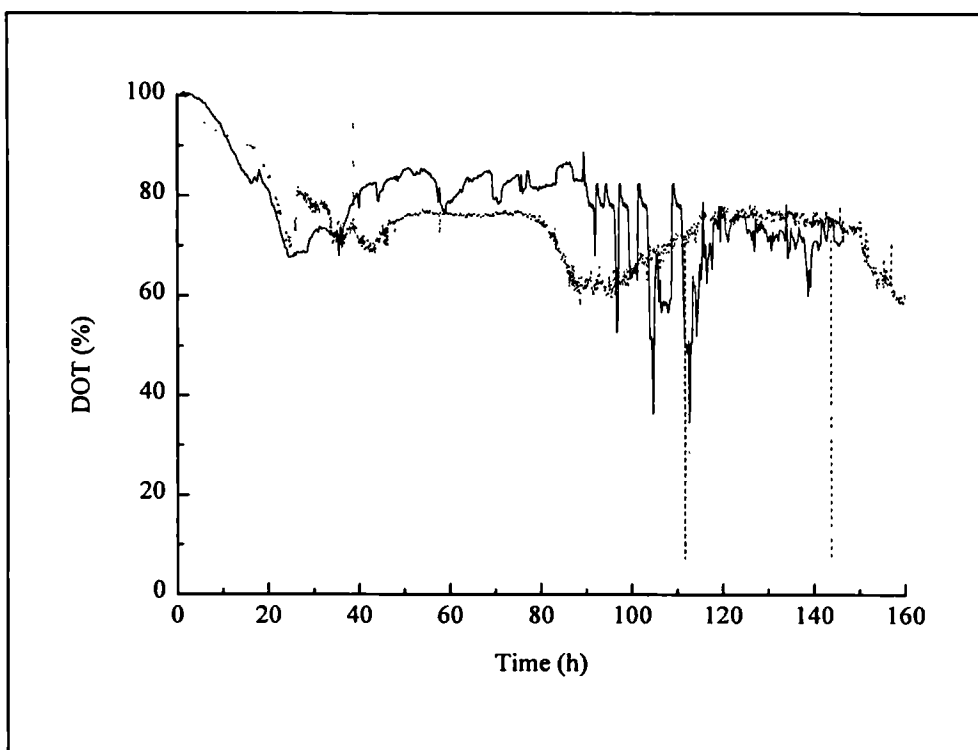


**Figure 6.2. Effect of 0.0% (■), 0.1% (●), 0.2% (▲), 0.3% (▼) and 0.5% (◆) SDS on growth of *S. clavuligerus***

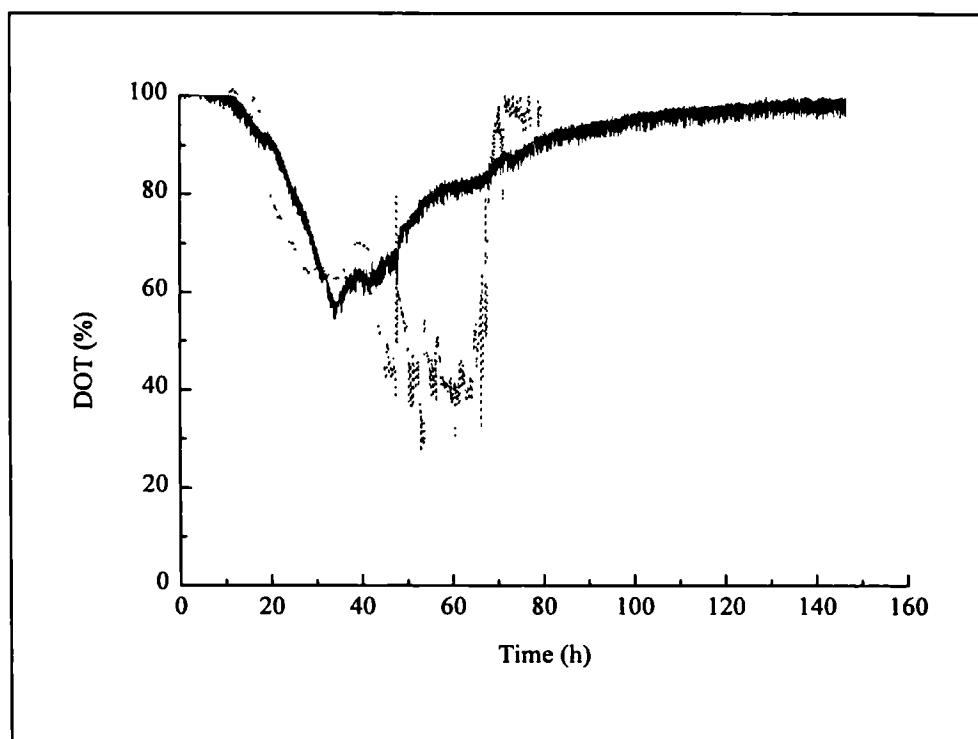
## **6.2. The effect of surfactant addition on growth**

### **6.2.1. Dissolved oxygen tension**

DOT was maintained above 40%, where necessary, by mixing oxygen into the air inlet whilst keeping total air flow at a constant 1 vvm. Figure 6.3. shows the data collected from fermentations containing 0.05%, 0.075% and 0.1% SDS and a control fermentation. The DOT for the control experiment and the fermentation containing 0.05% SDS were very similar with an initial decline, 0-20 h, to approximately 67%, Figure 6.3.a. The 0.05% SDS fermentation has a second decrease in DOT after 80 h. This may be attributed to a second growth phase. Profiles in Figure 6.3.b. are also similar to each other and the control and 0.05% SDS fermentation in the initial stages, 0-30 h, with a decrease to approximately 60%. After 30 h, DOT in the fermentation containing 0.075% SDS steadily increased. The DOT in the 0.1% SDS fermentation continued to decrease and was maintained above 40% by mixing oxygen with the air inlet. The extended decrease in DOT may be attributed to the increase in oil metabolism due to increased lipid dispersion. After 60 h there was a sharp increase in dissolved oxygen which may be due to exhaustion of the carbon substrate. The differences in growth between fermentations may also be due to the effect of SDS on oxygen transfer.



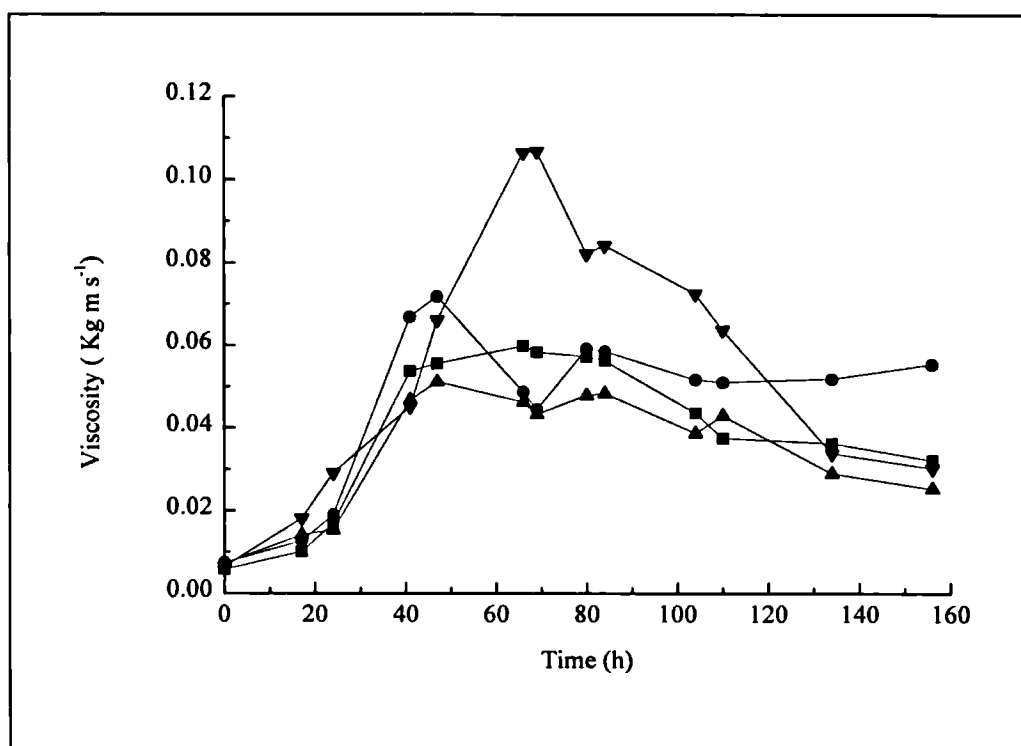
**Figure 6.3.a. DOT profile for fermentations containing 0% ( - ) and 0.05% ( ..... ) SDS.**



**Figure 6.3.b. DOT profile for fermentations containing 0.075% ( - ) and 0.1% ( ..... ) SDS.**

### 6.2.2. Viscosity

Differences in broth viscosity was used as a measure of changes in biomass levels, Figure 6.4. Readings at 0 h show the initial viscosity of the broth minus biomass. Data showed that SDS had little effect on initial growth, (0-40 h), of *S. clavuligerus* in 5 L batch fermentations. Fermentations containing 0.05% and 0.075% SDS showed similar trends in growth when compared to the control experiment with a maximum at 40 h, of between 0.05 and 0.07  $\text{Kg m s}^{-1}$ , and a slow decline towards 160 h. At the level of 0.1% the growth phase was extended with a maximum viscosity of 0.108  $\text{Kg m s}^{-1}$  reached after 70 h. The enhanced growth phase may be due to increased dispersion of the oil for utilisation of the lipid carbon source for growth. The increase was seen in the duplicate fermentation and corresponds with the decrease in DOT seen in Figure 6.3.b.



**Figure 6.4. Viscosity throughout fermentations containing 0% (■), 0.05% (●), 0.075% (▲) and 0.1% (▼) SDS.**

### 6.2.3. Capacitance

Initial bench top experiments were carried out to investigate the effect of addition of SDS on solution capacitance, Figure 6.5. A plot of the difference in capacitance between the media to be tested and a salt solution of similar conductance through a frequency scan gives an indication of the effect of SDS addition. Actual capacitance

decreased with increasing concentrations of SDS and therefore the capacitance difference between solutions decreased, Figure 6.5.a. SDS had no significant effect on media minus oil, Figure 6.5.b. Differences in capacitance may be due the oil collecting near the biomass probe pins. This effect would not account for the pattern seen with increasing concentrations of SDS. The capacitance fall may be due to the SDS increasing dispersion of the oil. Oil has a relative permittivity of less than 5 and water a relative permittivity of approximately 80. Thus dispersion of the oil into the water may reduce the total relative permittivity, Equations 6.1 - 6.4 (Weber *et al.*, 1952).

Capacitance (C) is the ratio of charge (Q) and potential difference between poles (V):

$$C = Q / V \quad (6.1)$$

Response of a medium to an electric field is characterised by the quantity of permittivity ( $\epsilon$ ) obtained from Coulomb's law, Equation 6.2. The magnitude of force between charges (F) is equal to the permittivity ( $\epsilon$ ) and the separation (s) between the charges Q and Q'.

$$F = (1 / 4 \pi \epsilon)(QQ' / s^2) \quad (6.2)$$

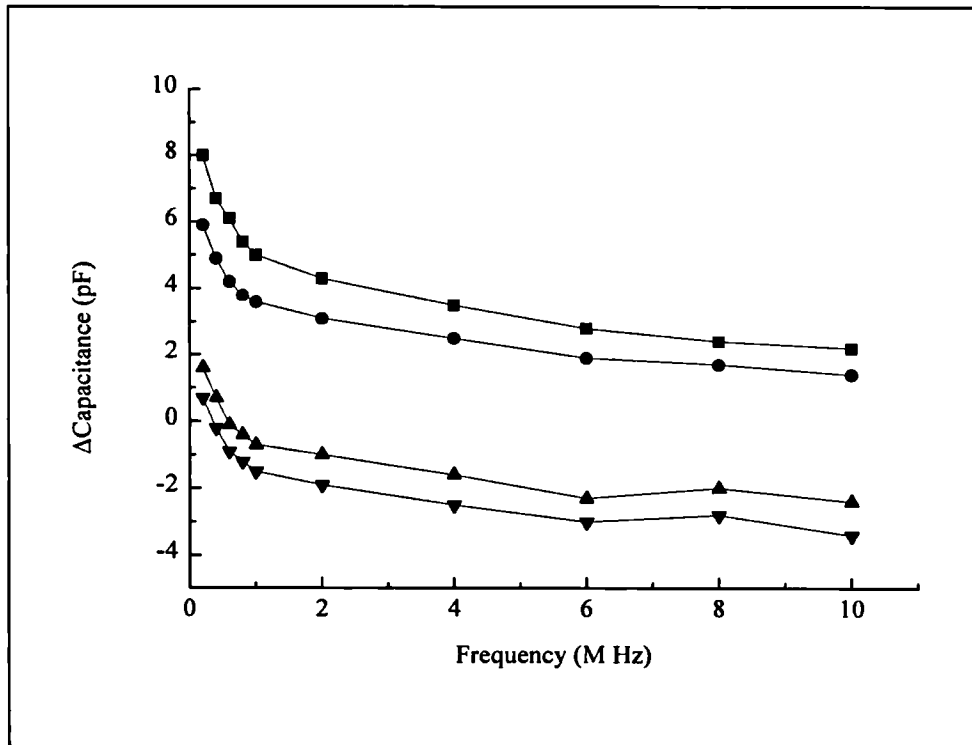
Potential difference of a uniformly charged spherical conductor, i.e. an oil droplet, will have the same charge as if all the charge were concentrated at the centre of the sphere of radius r. Potential difference is given by:

$$V = (1 / 4 \pi \epsilon)(Q / r) \quad (6.3)$$

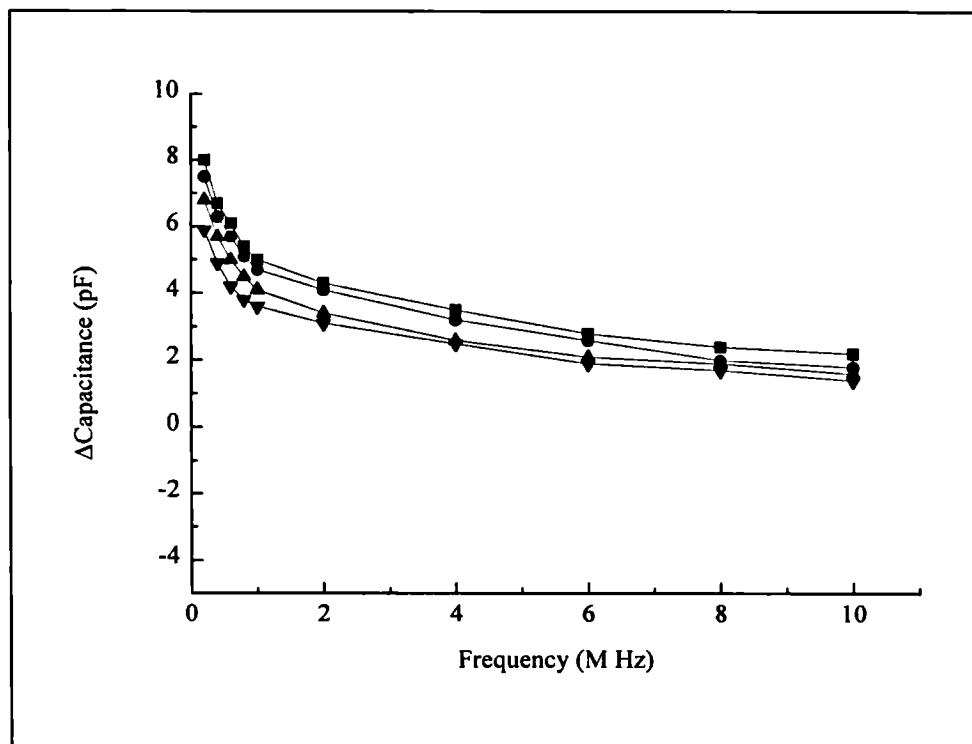
Therefore the capacitance of an isolated spherical conductor may be given as:

$$C = Q / V = 4 \pi \epsilon r \quad (6.4)$$

Hence the capacitance of a sphere is proportional to its radius. The actual capacitance of the media decreased on addition of surfactant. This may indicate a decrease in either the permittivity and/or radius of the oil droplet.



**Figure 6.5.a. Difference in capacitance of oil based media with addition of SDS at 0% (■), 0.05% (●), 0.075% (▲) and 0.1% (▼).**

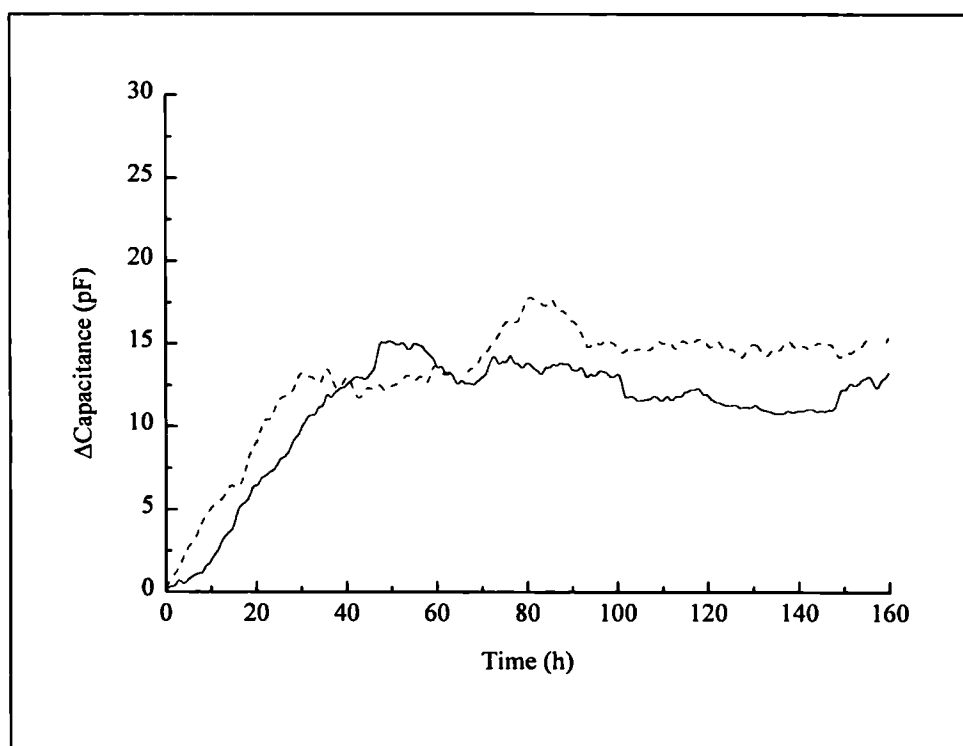


**Figure 6.5.b. Difference in capacitance of media minus oil with addition of SDS at 0% (■), 0.05% (●), 0.075% (▲) and 0.1% (▼).**

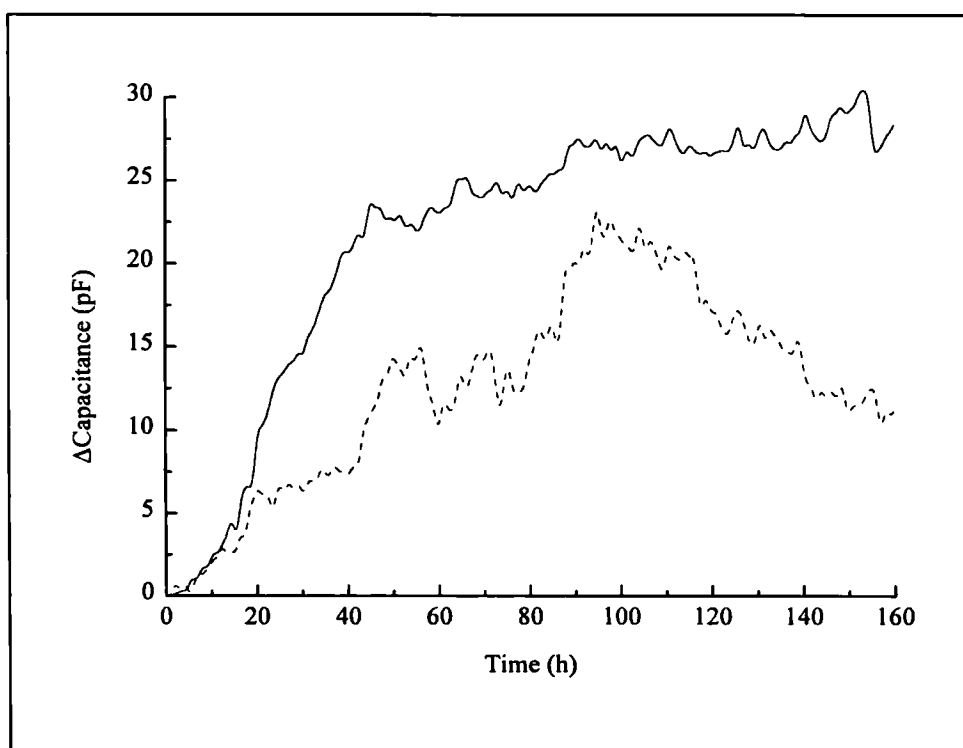


Differences in initial capacitance due to the addition of SDS to the media were accounted for in initial calibration of the biomass monitor and therefore do not affect capacitance readings throughout the fermentation. Capacitance data, Figure 6.6., showed that, when compared to the control, the fermentation containing 0.05% SDS was very similar as suggested by previous viscosity, Figure 6.4, and DOT data Figure 6.3.. There was a significant difference between capacitance measurements of fermentations held at 0.075% and 0.1% SDS, compared to the control experiment. All fermentations containing SDS were similar in the initial stages, 0-20 h. The fermentation containing 0.075% SDS increased to a maximum capacitance of 22.5 pF at 45 h, after which the capacitance steadily increased to a final value of approximately 27 pF. This was a high value when compared to the fermentation containing 0.05% SDS and the control which initially peaked at 37 h and 50 h with capacitance readings of 12.5 pF and 15 pF respectively. The fermentation containing 0.05% SDS had a second peak at 80 h which corresponds to the second decrease in DOT seen in Figure 6.3.a. and was attributed to a second growth phase.

Growth measurements using viscosity data, Figure 6.4., suggested that the fermentation containing 0.1% SDS was the most successful in terms of biomass production. This was not supported by capacitance data, Figure 6.6.b. For the 0.1% SDS fermentation biomass monitor data showed that there was an initial increase in capacitance, 0-20 h, as in all SDS fermentations discussed, after which there was a lag of 20 h before a further increase in capacitance. This was not shown in off-line viscosity measurements, even in samples taken at 23 h. The delay may be due to the mycelia blocking the biomass probe pins or there is an actual delay in the growth phase. After the next increase in capacitance at 40 h there was a further delay in capacitance until another increase at 80 h. The maximum capacitance reached is 22 pF, higher than in the fermentations containing 0.05% SDS and the control experiment. After 100 h there was a decrease in capacitance to a final level of 10.5 pF. The staged growth was not shown in viscosity analysis and may be due to mycelial blocking of the probe pins as the viscosities reached are higher than in any other fermentation. Capacitance data does also not correspond with on-line DOT data with the 0.1% SDS fermentation.



**Figure 6.6.a. On-line  $\Delta$ capacitance measurements during a batch cultivation containing 0.0% ( - ) and 0.05% SDS ( --- ).**

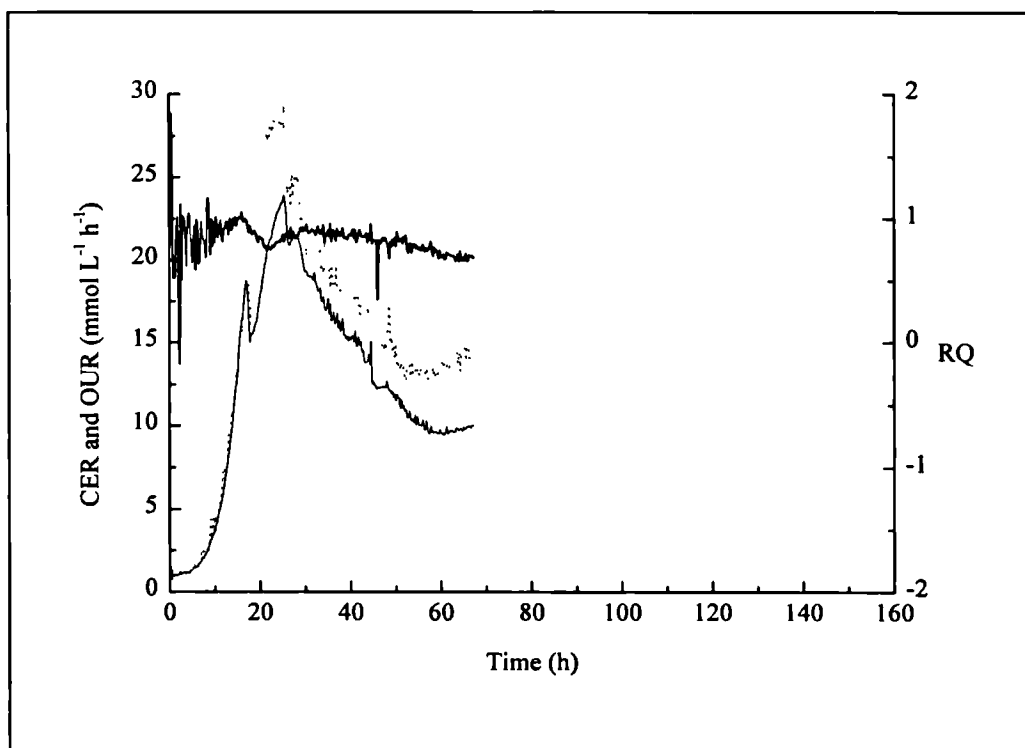


**Figure 6.6.b. On-line  $\Delta$ capacitance measurements during a batch cultivation containing 0.075% ( - ) and 0.1% SDS ( --- ).**

#### 6.2.4. Exit gas analysis

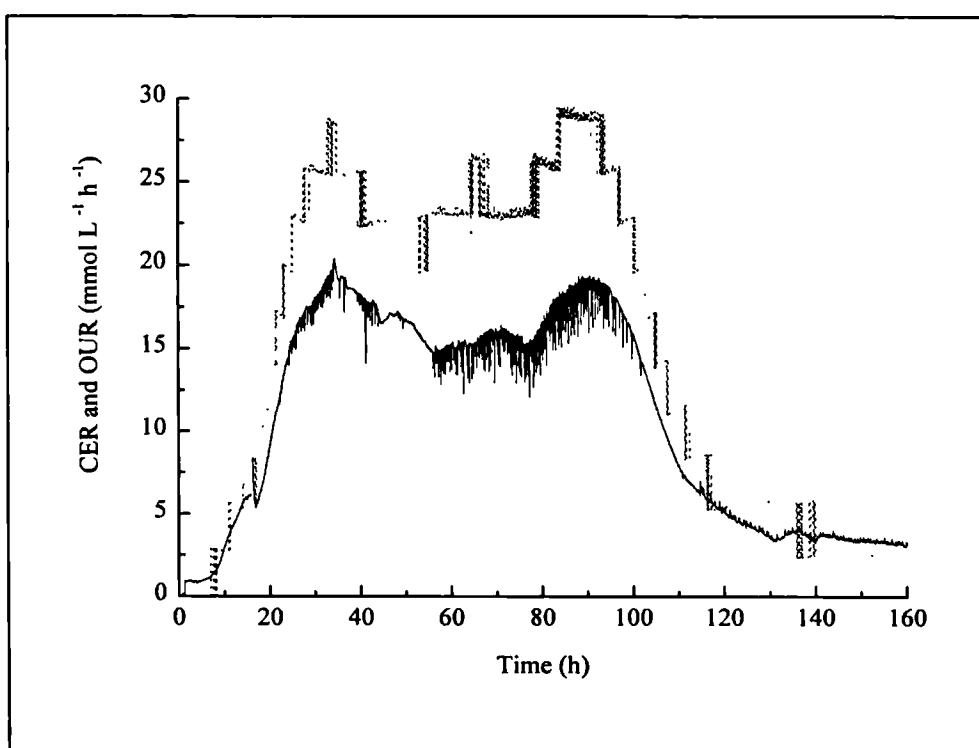
Exit gas analysis is not complete due to errors in data logging and mass spectrometry. Data reported is for the control experiment, Figure 6.7.a. and the average of duplicate fermentations containing 0.05% SDS, Figure 6.7.b. Although exit gas is incomplete for the control fermentation there is a significant difference between fermentations presented. As with all previous media comparison and stirrer speed fermentations the control experiment exit gas data showed a single peak at approximately 20-30 h, depending on fermentation conditions, after which there was a steady decline in carbon dioxide evolution and oxygen uptake. This corresponds to the measurement of biomass through viscosity and capacitance for the control fermentation.

In the fermentation containing 0.05% SDS there are two peaks in exit gas analysis. The stepped OUR data is due to manual calculation. The CER and OUR initially increased from 0-40 h, to 20 and 27 mmol L<sup>-1</sup> h<sup>-1</sup> respectively. The second peak can be seen after 80 h and is similar in quantity to the initial peak. The second peak corresponds to the decrease in DOT seen in Figure 6.3.b. and the increase in capacitance, Figure 6.6.a. There is also a two peak profile seen in biomass measurements through changes in media viscosity, Figure 6.4. Two peaks may be attributed to a two phase growth. The initial peak may be due to growth on the carbohydrate carbon source and the smaller oil droplets. The second peak is most likely a result of the metabolism of rapeseed oil due to emulsification and stabilisation of oil droplets by SDS. Although exit gas analysis is incomplete viscosity data showed that the two phase growth does not seem to occur in fermentations containing higher concentrations of SDS. This may be due to more rapid emulsification of the rapeseed oil with the increased concentration of surfactant.



**Figure 6.7.a. Exit gas analysis for control experiment.**

( --- OUR — CER, — RQ )



**Figure 6.7.b. Exit gas analysis for fermentation containing 0.05% SDS**

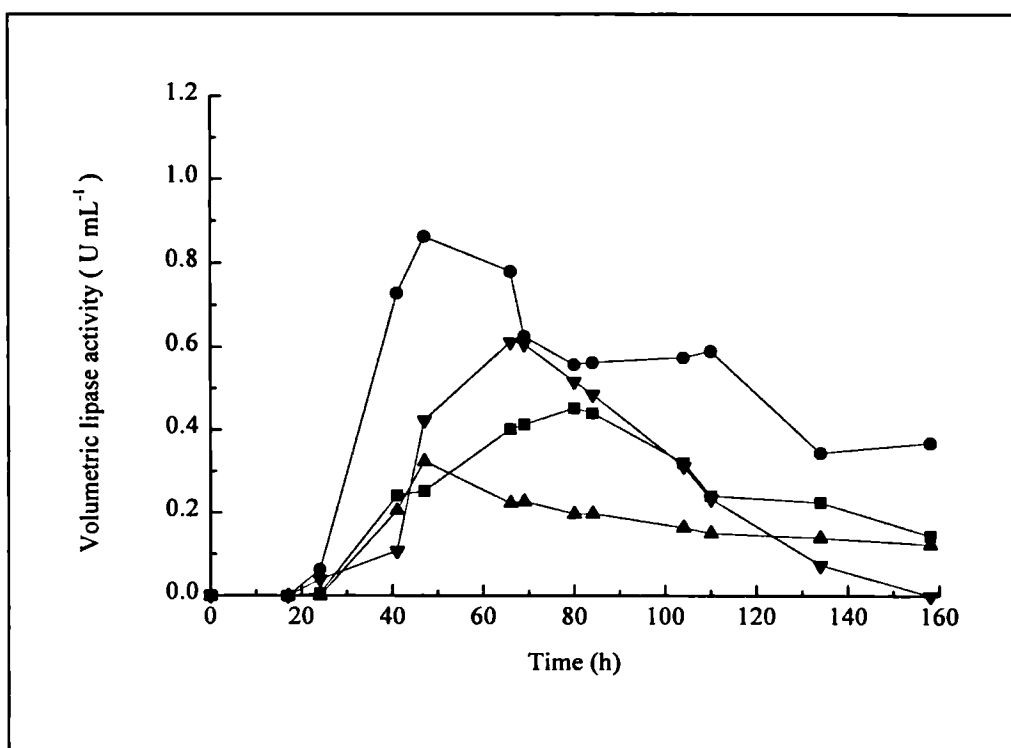
( --- OUR — CER, )

### **6.3. Lipase activity**

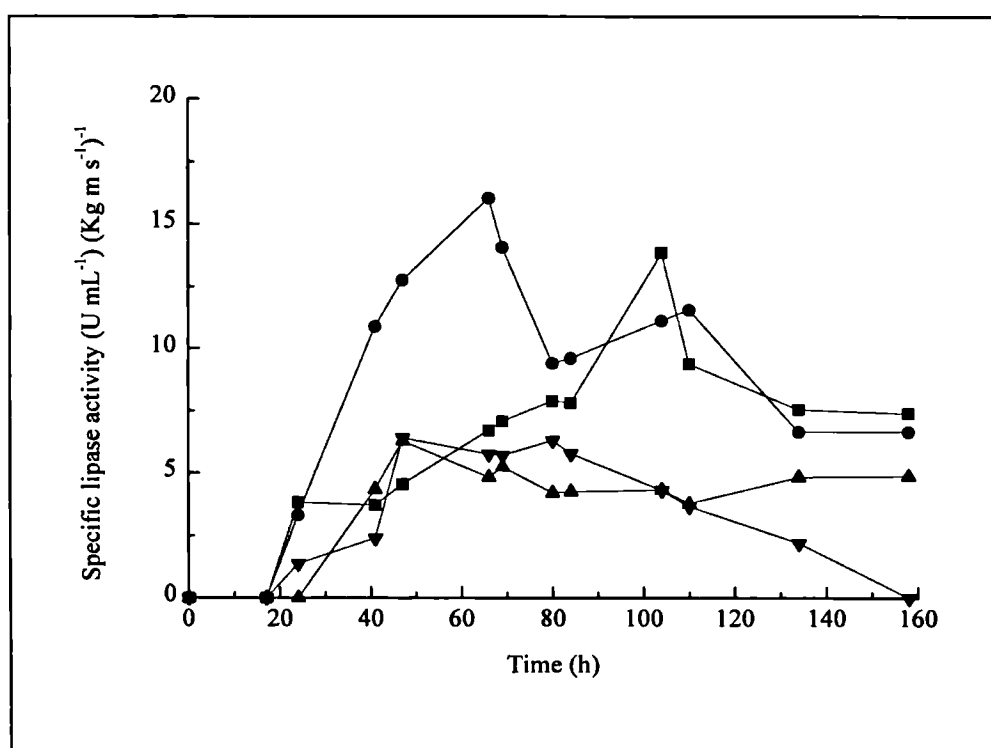
Since the lipase reaction is reported to occur at the oil-water interface the rate of hydrolysis is a direct function of interfacial area (Choo and Lee, 1989; Macrae, 1989; Marangoni, 1994). Shiomori *et al.* (1995) concluded that the rate of lipase hydrolysis of olive oil in a monodispersed emulsion was affected by the concentration of the olive oil and lipase, the interfacial area and droplet diameter of the emulsion. SDS was added to the process media with the aim of emulsifying the rapeseed oil and stabilising drop size. Addition of SDS may increase interfacial area and hence increase lipase activity.

Volumetric lipase activities for fermentations with increasing concentrations of SDS are shown in Figure 6.8. Average results from duplicate fermentations are plotted. Relative standard errors between fermentations, not plotted, were less than 5 %. Highest volumetric lipase activity was seen when SDS was added at a concentration of 0.05%. There are two peaks detected in lipase activity which correspond to peaks in biomass analysis for fermentations held at 0.05% SDS. When the level of SDS was increased to 0.1% detected lipase activity fell. This may be attributed to the increase in surfactant molecules shielding the lipase enzyme from the site of action, the interface. It may also be due to the fact that SDS is an anionic surfactant used in the solubilisation of proteins. The surfactant may have solubilised some of the lipase enzyme or, as the lipase in *S. clavuligerus* may be associated with the membrane, the SDS may have interfered with enzyme/membrane activities. Detected lipase activity in the fermentation containing 0.1% SDS decreases to zero. This may be due to the loss of activity due to the solubilisation of protein or due to the lack of lipid substrate, Section 6.4. At a concentration of 0.075% SDS lipase activity is less than detected in the control experiment. To eliminate differences in detected activities due to biomass levels specific lipase activities were calculated.

As with previous chapters viscosity was used as a measure of biomass to calculate specific lipase activity. Specific lipase activity was increased by the addition of 0.05% SDS when compared to the control, Figure 6.9. At higher concentrations of SDS, 0.1% and 0.075%, specific lipase activity was reduced when compared to the control, as shown by volumetric lipase activities.



**Figure 6.8.** Volumetric lipase activity in fermentations containing 0% (■), 0.05% (●), 0.075% (▲) and 0.1% (▼) SDS.

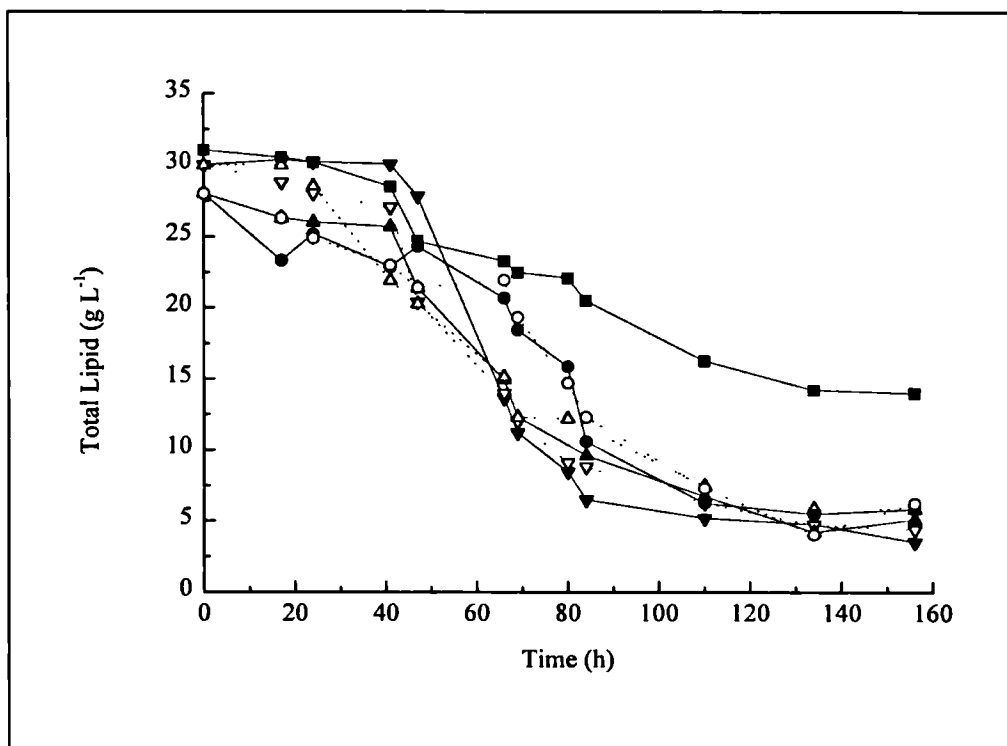


**Figure 6.9.** Specific lipase activity in fermentations containing 0% (■), 0.05% (●), 0.075% (▲) and 0.1% (▼) SDS.

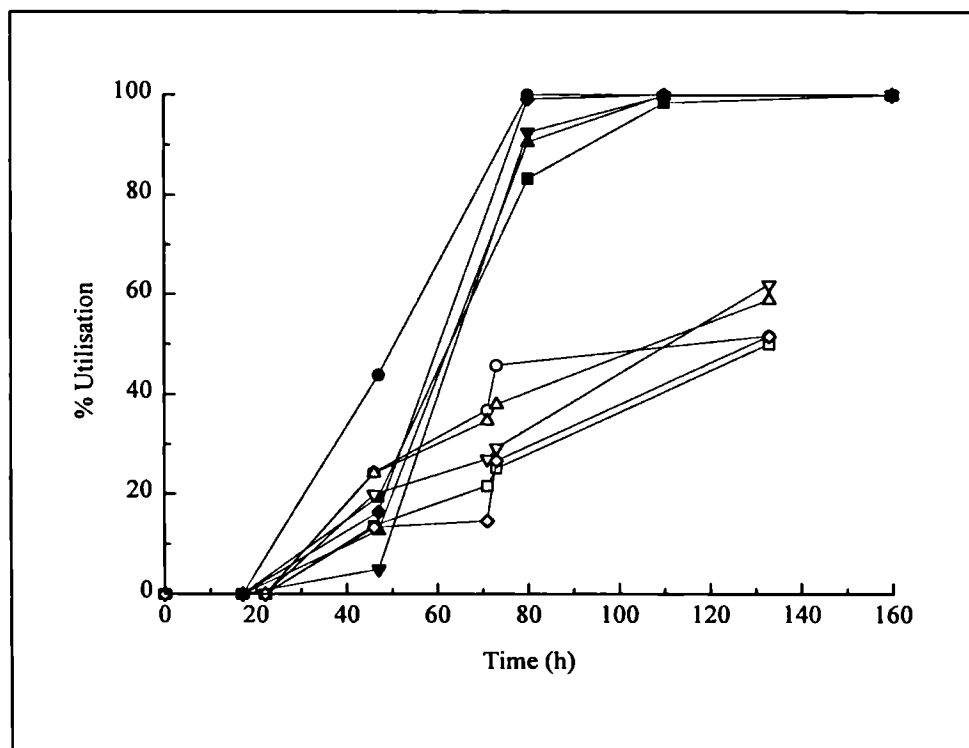
#### **6.4. Total lipid analysis**

Total lipid analysis, using the vanillin total lipid assay, for all SDS comparison fermentations are shown in Figure 6.10. Results from duplicate fermentations were plotted. Data revealed a dramatic decrease in the residual oil levels in fermentations containing SDS when compared to the control. Initial total lipid levels were higher than previous fermentations due to the increase in lipid based antifoam. Initial lipid levels, 0-40 h, remained high in all SDS fermentations. It was noted that addition of SDS to samples and standards reduced standard deviations. SDS stabilised the droplets within samples and reduced errors in sample preparation. SDS could be used in future total lipid assay method development. In all fermentations there was a rapid decrease in total lipid level between 40 h and 80 h, after which the level of detected lipid levels to approximately 20% of the initial lipid added. This is a significant decrease compared to the residual oil level of the control, 50% of the input oil. The residual oil level is higher in the control in this experiment than previous fermentations due to antifoam interfering with the total lipid assay. Residual oil levels in the SDS fermentations may be due to the inability to use the lipids from the antifoam.

Gas chromatography of whole broth samples throughout the SDS comparison fermentations were analysed to identify fatty acids present in the residual oil, Figure 6.11. This will eliminate total lipid results due to antifoam addition as the antifoam contains different fatty acids to the rapeseed oil. Chromatographic analysis of standards containing SDS revealed that the efficiency of the method decreased from approximately 98% to 80%. The surfactant may affect separation through interference with the column packing. Peak definition was still good. Gas chromatography results were therefore plotted as a percentage utilisation from initial input oil as the surfactant effects on chromatography were the same throughout the duration of the fermentation. There is a need to develop a more accurate chromatographic method or investigate packing materials which will not be affected by surfactants. Gas chromatography revealed that the fatty acids present in rapeseed oil were not detected in whole broth samples from fermentations containing 0.05-0.1% SDS after 100 h fermentation. There was also little apparent selectivity towards the individual triglycerides of rapeseed oil. The effects of increasing concentrations of SDS on the utilisation of individual fatty acids are shown in Appendix II.



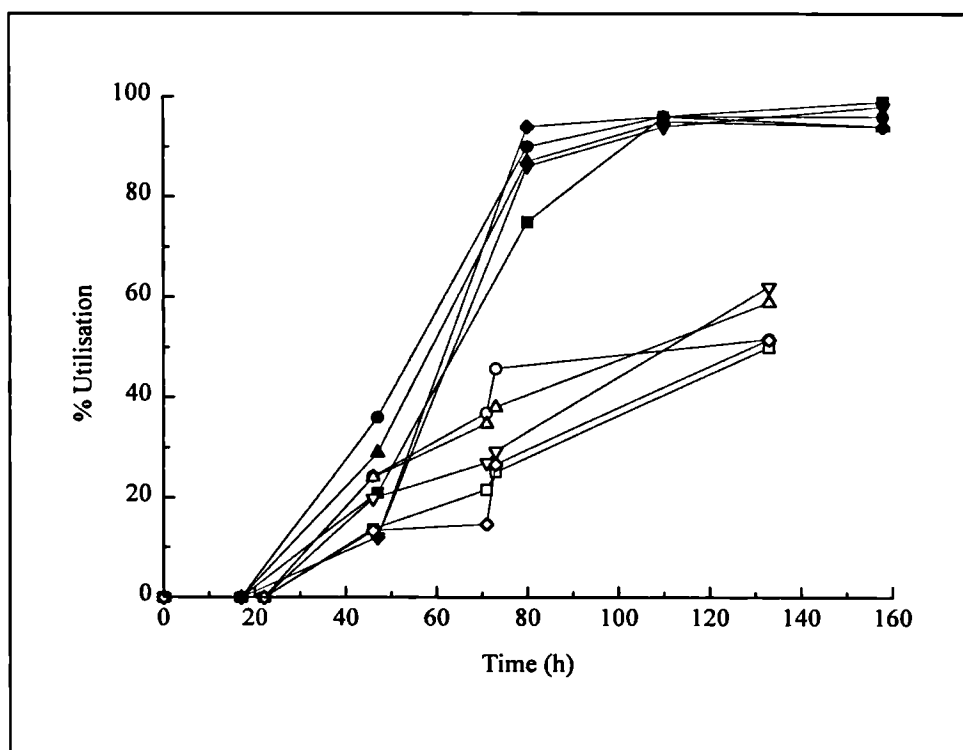
**Figure 6.10.** Total lipid analysis for fermentations containing at 0% (■), 0.05% (●), 0.075% (▲) and 0.1% (▼) SDS.



**Figure 6.11.a.** Fatty acid utilisation in a fermentation containing 0.0% (□) and 0.1% (■) SDS.

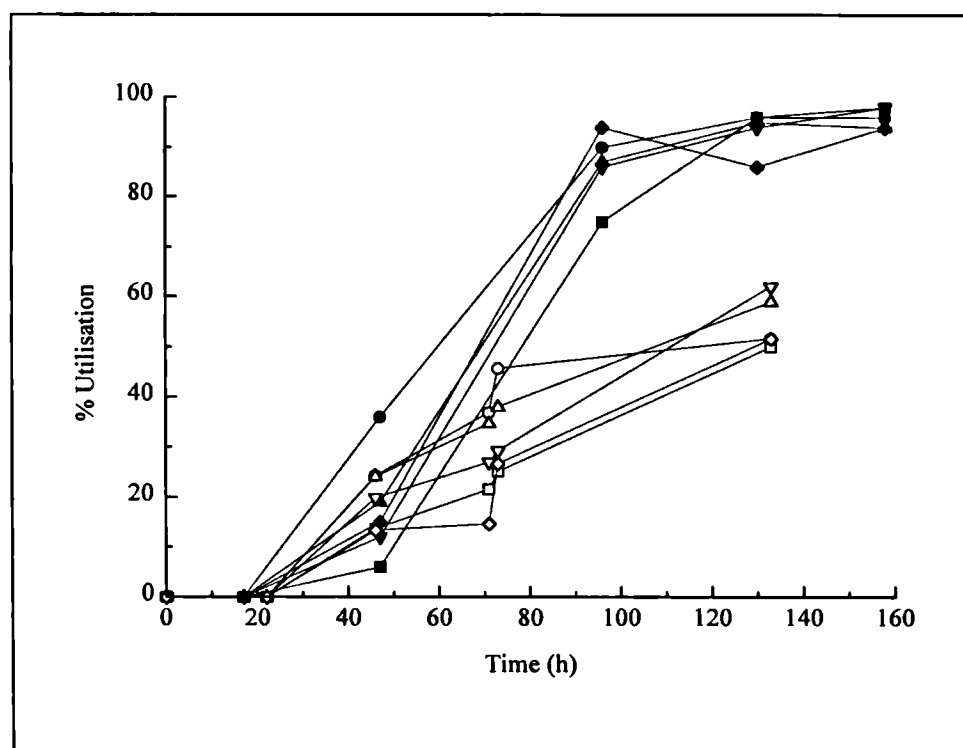
*Palmitic* (■), *Stearic* (●), *Oleic* (▲), *Linoleic* (▼) and *Linolenic acid* (◆).





**Figure 6.11.b. Fatty acid utilisation in a fermentation containing 0.0% ( □ ) and 0.075% ( ■ ) SDS.**

*Palmitic ( ■ ), Stearic ( ● ), Oleic ( ▲ ), Linoleic ( ▼ ) and Linolenic acid ( ◆ ).*



**Figure 6.11.c. Fatty acid utilisation in a fermentation containing 0.0% ( □ ) and 0.05% ( ■ ) SDS.**

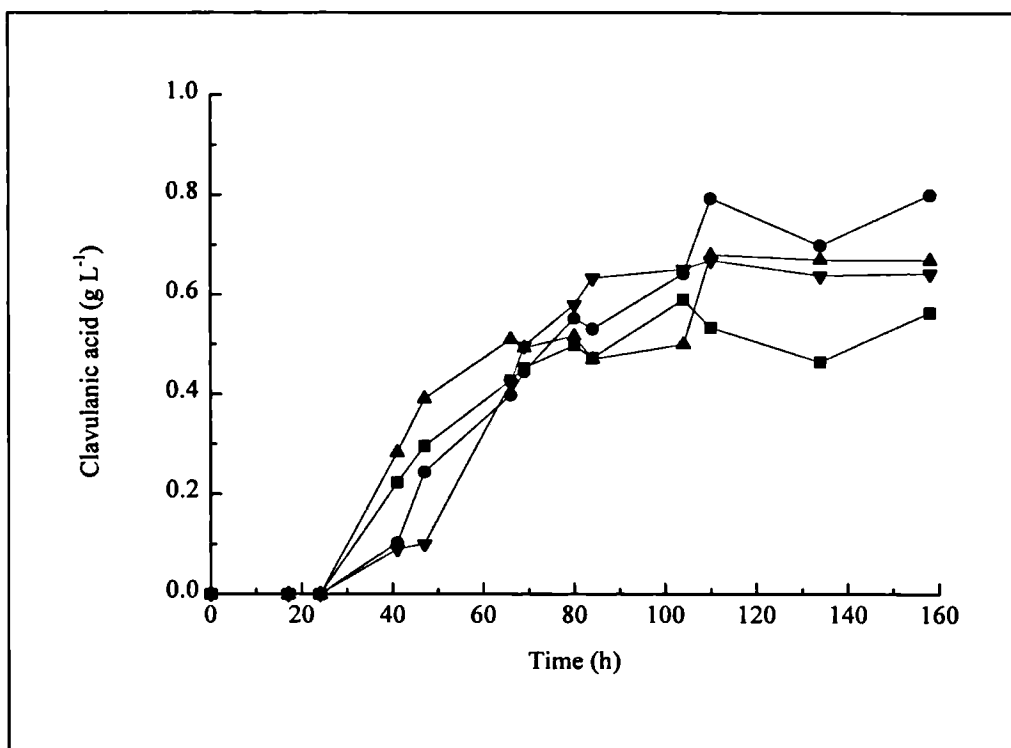
*Palmitic ( ■ ), Stearic ( ● ), Oleic ( ▲ ), Linoleic ( ▼ ) and Linolenic acid ( ◆ ).*

### **6.5. Clavulanic acid**

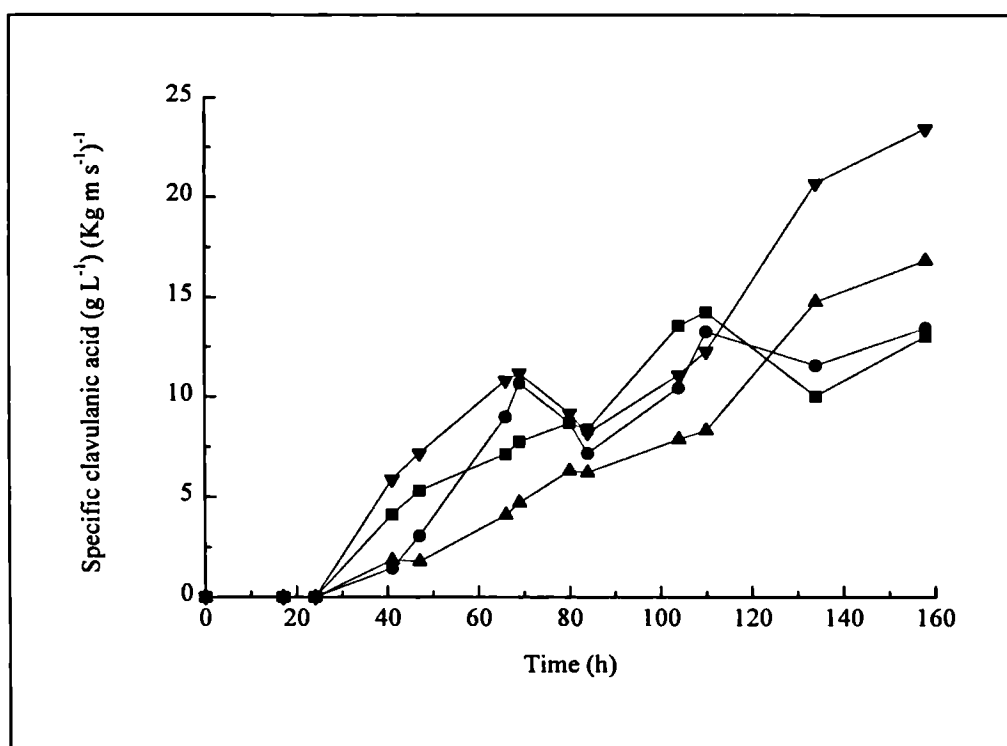
Total lipid analysis and gas chromatography of whole broth samples revealed a dramatic increase in lipid utilisation. As the addition of rapeseed oil enhanced antibiotic titres, Section 4.2., an increase in lipid utilisation would also be expected to raise clavulanic acid titres. Volumetric clavulanic acid titres for the fermentations containing SDS, Figure 6.12, show a notable increase in final product values. The control fermentation had a final titre of  $0.56 \text{ g L}^{-1}$  compared to the 0.05%, 0.075% and 0.1% SDS fermentations showing final titres of 0.80, 0.67 and  $0.63 \text{ g L}^{-1}$  respectively. Due to the differences in biomass levels a more accurate comparison of productivity can be made through specific clavulanic acid titres, Figure 6.13.

Using viscosity as a measure of biomass specific productivity profiles revealed that throughout the initial stages of fermentation, 0-80 h, the addition of 0.1% SDS increased productivity, 0.075% decreased titres and 0.05% was similar to the control. At 80 h all specific clavulanic acid titres were approximately equal. After 160 h the data showed an approximate two fold increase in clavulanic acid titre in fermentations containing 0.1% SDS when compared to the control. Although many factors affect media viscosity including hyphal morphology and media constituents it is the only method available for comparison of growth in a process containing such complex media. The increase in titre may be due to the increase in lipid utilisation. Lipid utilisation in the 0.05% SDS fermentation was greatly enhanced compared to the control experiment and final clavulanic acid titres are very similar. Final product titres were increased from 13 to  $17.5 \text{ (g L}^{-1}) \text{ (Kg m s}^{-1})^{-1}$  when the concentration of SDS in the process media was increased from 0.05% to 0.075%, and increased further to  $23.5 \text{ (g L}^{-1}) \text{ (Kg m s}^{-1})^{-1}$  with addition of 0.1% SDS. Lipid utilisation and residual oil levels were approximately equal in all SDS fermentations. SDS did not affect the chromatography of clavulanic acid standards. Final viscosities were low in the 0.1% SDS fermentation which explains the high specific productivity.

The addition of SDS to the fermentation media not only acts as a surfactant and emulsifier but increases the level of sulphate available to the organism. The pattern of raised specific productivity with increasing levels of SDS, as lipid utilisation remains the same, may be as a result of increase sulphate present in the media.



**Figure 6.12.** Volumetric clavulanic acid titres for fermentations containing 0% (■), 0.05% (●), 0.075% (▲) and 0.1% (▼) SDS.



**Figure 6.13.** Specific clavulanic acid titres for fermentations containing 0% (■), 0.05% (●), 0.075% (▲) and 0.1% (▼) SDS.

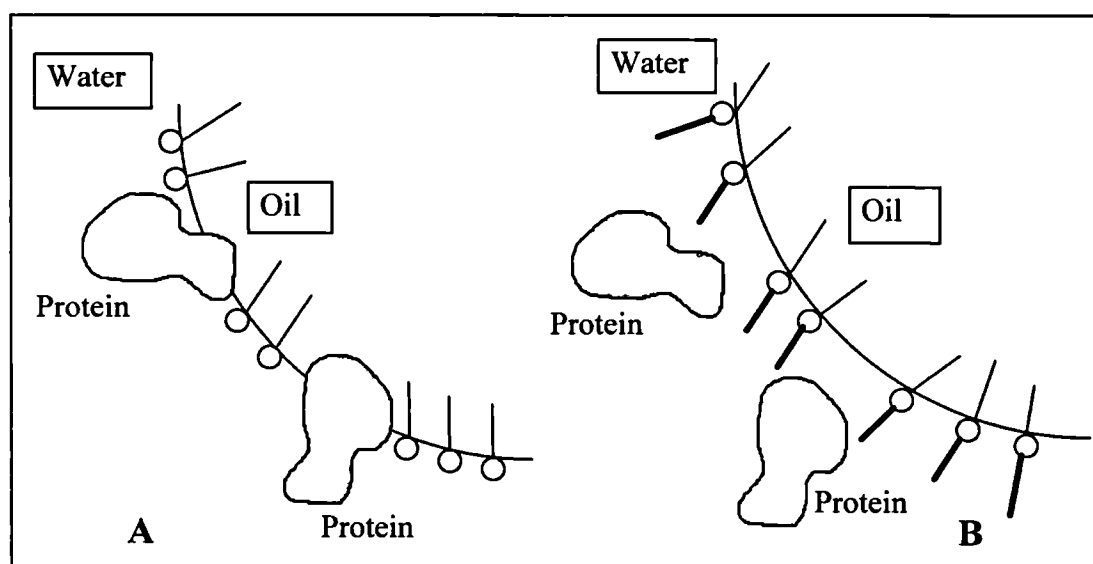
## **6.6. Discussion**

Increasing tip speed in a 5 L batch fermentation of *S. clavuligerus* did not increase lipid utilisation. It was concluded that this may be due to the inability of the organism to utilise the oil as the lipid remained in large droplets. SDS was added to fermentation media with the aim of increasing lipid dispersion and reducing oil droplet size. *In situ* particle size analysis would be necessary in order to determine optimum concentrations of surfactant and tip speed needed to decrease and stabilise oil droplet size. The concentration of surfactant would then be compared to the effects on growth of *S. clavuligerus* to determine the optimum concentration to be added to the fermentation. The concentration of SDS to be added to the process media is critical to adequate lipid dispersion and growth. It has previously been noted by Shiomori *et al.* (1995) that a concentration of lower than 0.1% SDS induced an increase in droplet diameter and in droplet diameter distribution in olive oil emulsions.

Experiments were carried out to investigate the effects of 0.05%, 0.075% and 0.1% SDS on 5 L batch fermentations containing complex process media. Changes in biomass, measured through viscosity, seemed relatively unaffected by changes in concentration of SDS with the exception of the highest concentration, 0.1%. Measurement of capacitance throughout the duration of the fermentation was inconclusive at higher SDS concentrations. This was attributed to the high viscosity of the whole broth. 0.075% SDS increased fermentation capacitance when compared to a control. Capacitance data also showed the relationship between permittivity and oil droplet radius with the concentration of SDS. DOT data showed that increasing the concentration of SDS increased growth. The increase in growth may be due to the increase in lipid utilisation through enhanced dispersion of the oil droplets.

Lipase activity does not seem to be a limiting factor in the utilisation of the rapeseed oil in 5 L batch fermentations of *S. clavuligerus*. This was indicated by the varying levels of detected lipase activity compared with constant lipid utilisation profiles seen in previous media comparison and tip speed fermentations. However, to use the lipase activity effectively in the fermentation containing complex media a fundamental understanding of the role of substrates, products and the site of catalysis are required.

Data presented showed that the addition of 0.05% SDS to the process media increased detected lipase activity. Increasing the SDS concentration further decreased detected lipase activity. It has been reported that surfactants and dispersion stabilising agents used in emulsion preparation can affect lipase activity in complex ways (Kawase *et al.*, 1985). The type of surfactant used will influence the type of emulsion formed and the activity of the lipase. The majority of research has centred around the use of anionic surfactants such as sodium bis (2-ethylhexyl)sulfosuccinate and SDS. Lipase catalysed hydrolysis is rapid in microemulsions based on anionic surfactants when compared to the more sluggish activities observed when the same concentration of nonionic surfactants are used (Skagerlind and Holmberg, 1994). The difference may be attributed to accessibility of the triglyceride to the enzyme, Figure 6.14.



**Figure 6.14. A proposed arrangement at the oil-water interface for lipase activity in microemulsions based on anionic surfactant (A) and nonionic surfactant (B). (o) hydrophilic head, (-) lipophilic tail and (—) side chains of surfactant molecule. (Skagerlind and Holmberg, 1994)**

Lipase activity occurs at the oil-water interface. A change in the surface properties of the substrate caused by adsorption of surfactants will affect lipase hydrolysis. Shielding of the interface by additional chains or increased concentration of surfactant may obstruct access to the interface between the oil and water domains. As SDS is a stabilising agent more than an emulsifier increasing the concentration of the surfactant may have shielded the interface rather than increasing the interfacial area through emulsification. Both nonionic and anionic surfactants reduced lipase activity with

increased surfactant concentration when tested in a number of organisms (Kawase *et al.*, 1985). Measurements of kinetic parameters using SDS concluded that the change in surface property (charge) caused by the adsorption of surfactants indicated competitive inhibition of lipase hydrolysis. It may be that the sulphate groups of SDS adsorbed on the emulsion surface influence the active site of lipase. An explanation for the increase in detected lipase activity on addition of 0.5% SDS may be that the anionic surfactant may interact with the lipase protein and produce a lipase-surfactant complex which could act on the substrate and stimulate lipase hydrolysis. Lipase activity decreased with higher concentrations of SDS which may affect the active point of lipase hydrolysis and produce inhibition. However, nonionic surfactants have been shown to activate lipase with a rise in concentration (Kawase *et al.*, 1985). A conformational change as discussed with the anionic surfactant would not occur until higher concentrations because of weak electrostatic interactions between substrate, enzyme and active point of lipase. Results by Kawase *et al.* (1985) show that mixing nonionic and ionic surfactants protects the decrease in lipase activity. This method may be valuable in the process media to enhance lipase activity whilst increasing oil dispersion.

Previous research has shown that when a phosphate buffer was used as a polar component instead of water the rate of hydrolysis of a lipase on palm oil was considerably increased (Skagerlind and Holmberg, 1994). This was attributed to the differences in charge at the interface between the oil and water domains. The shielding effect of the counter ions on the negative charges of the surfactant may facilitate access of the lipase to the interfacial region. Addition of salts in combination with surfactants could be investigated in order to determine the effect on lipase activity and its relationship to lipid utilisation in the fermenter environment.

On-line measurements of the interfacial area of emulsions in order to study lipase kinetics have been investigated. Static analysis of the reactions are available using microscopic techniques although detergents are usually added to stabilise the emulsion. Kierkels *et al.* (1990) used an optical particle sizer, based on light scattering, to measure directly the droplet size distribution during a lipase catalysed reaction. From this droplet distribution the total interfacial area of the emulsion could be calculated and determination of kinetic parameters possible. This may be possible in a more defined media with less complex particles.

Alternatively the decrease in detected lipase activity with increasing concentrations of SDS may be due to the increase in solubilisation of the protein itself. As the lipase activity in *S. clavuligerus* has been reported to be associated with the membrane of the mycelia (Cavanagh, 1996) the surfactant may also affect the interaction of the protein with the membrane and/or fatty acid transport in to the cell.

Total lipid analysis and gas chromatography data showed that addition of the surfactant to the process media significantly enhanced lipid utilisation and reduced residual oil levels. A model was proposed by Koshy *et al.* (1988) for the effect of surfactants on drop breakage in turbulent liquid dispersions. It was concluded that droplet breakage in stirred dispersions was influenced by the presence of the surfactant not only through reduction of the interfacial tension but also by generation of interfacial tension gradient across the droplet. This results in extra stress which adds to the turbulent stress and hence results in the formation of smaller droplets. The increase in lipid utilisation can be explained by a decrease and/or stabilisation of droplet size due to the addition of the surfactant, SDS.

Volumetric clavulanic acid data showed an increase in titre on addition of SDS when compared to the control. Specific clavulanic acid data, using viscosity as a measure of changes in biomass, showed that at 0.1% SDS the specific productivity of *S. clavuligerus* increased approximately two fold when compared to a control experiment. Viscosity measurements were much lower during the final stages of these fermentations when compared to other SDS experiments. This may contribute to the observed increase in specific productivity. Addition of SDS to the fermentation does increase final product titres. This may be due to the increase in lipid utilisation. Lipid utilisation was approximately equal in all SDS fermentations and clavulanic acid titres vary considerably. The difference in titres may be due to the level of sulphate and/or sodium present in the fermentation media. The sodium and/or sulphate increase may affect the enzymes involved in clavulanic biosynthesis although extensive literature reviews of the chemistry and biosynthesis pathway have revealed a comprehensive understanding of the chemistry involved with no sulphate or sodium control (Baggaley *et al.*, 1997; Townsend and Ho, 1985; Pitlik and Townsend, 1997; Elson and Oliver, 1978; Thirkettle *et al.*, 1997). The sodium and/or sulphate may affect gene expression from the clavulanic acid cluster present in *S. clavuligerus*. Review of present research in the

genetics of clavulanic acid expression (Ward and Hodgson, 1993; Paradkar and Jensen, 1995; Doran *et al.*, 1990; Aidoo *et al.*, 1994; Hodgson *et al.*, 1995; Jensen *et al.*, 1993) reveal that there is still limited information on the control of gene expression.



### **6.7. Summary of the effects of surfactant addition on lipid utilisation and clavulanic acid titres.**

The addition of SDS at concentrations of 0.075% and 0.1% increased biomass levels measured by capacitance and changes in broth viscosity.

At 0.05% concentration of SDS a two stage growth pattern was observed. The first stage may be due to initial growth on the carbohydrate and smaller oil droplets. The second growth stage may be a result of metabolism of the rapeseed oil remaining due to delayed dispersion of the oil at the lower concentration of surfactant.

Capacitance measurements can be used to determine changes in medium permittivity and droplet radius. Data recorded showed that increasing the concentration of SDS decreased the difference in capacitance between the media and a salt control. This may be as a result of a decrease in oil droplet size or a decrease in media permittivity. Both indicate an increase in emulsification of the rapeseed oil.

Lipase activity was increased with addition of 0.05% SDS and decreased with further increases in SDS concentration when compared to a control fermentation. This was attributed to either changes at the interface or an affect on the protein itself.

Lipid utilisation was increased and residual oil levels dramatically reduced on addition of SDS to the fermentation media. Gas chromatography results suggest total utilisation of fatty acids present in rapeseed oil. However the chromatographic assay may be affected by surfactant addition.

Volumetric and specific productivity data revealed that final clavulanic acid titres increased on addition of SDS. The raised titres may be due to the increase in lipid utilisation. Alternatively an increase in the sulphate and/or sodium content of the media may affect the chemistry and /or genetic expression involved in clavulanic acid biosynthesis.

## **7. CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK**

The aims and objectives of this research project were to investigate the effects of process parameters on lipid utilisation in a 5 L batch fermentation of *Streptomyces clavuligerus*. Process parameters investigated included the effect of varying carbon and nitrogen sources in the complex process media, the effect of increasing tip speed and the addition of a surfactant. The parameters were investigated with respect to lipid utilisation and residual oil levels, lipase activity and clavulanic acid titres.

### **7.1. Conclusions**

1. Addition of rapeseed oil, in combination with modified starch, as carbon source in the complex media increased final clavulanic acid titres by 900% when compared to a carbohydrate only based media. Residual oil levels at the end of the fermentation were approximately 30% of the initial input oil. The aim of this research project was to raise productivity by investigating process parameters affecting lipid utilisation and residual oil levels.
2. Lipase activity was only detected when oil was present in the fermentation media. There was an initial delay in detected activity of 24 h. Lipid utilisation and residual oil levels were unaffected by different levels of lipase activity. It is concluded that, although essential for lipid metabolism, the rates of lipid utilisation and the residual oil levels were not affected by changes in lipase activity.
3. The type and concentration of soya flour used in the process media significantly affected lipase activity. This was attributed to the differences in the level of protein within the flour.
4. A change in concentration of the soya flour affected the rate of lipid utilisation. The difference in rates of utilisation may be due to flour particle and oil droplet distribution. The dispersion of particles and oil droplets within the fermenter environment may be the key to increasing lipid utilisation and reducing residual oil levels.

5. Shake flask fermentations, using specific triacylglycerides and methyl esters of rapeseed oil, showed little difference in growth. Gas chromatography analysis of fermentation samples revealed no selectivity between the individual fatty acids of rapeseed oil. The composition of the residual oil was found to be the same as the initial input oil and is therefore not due to selective metabolism of the initial rapeseed oil.

6. Tip speeds of 1.88, 2.36, 2.83, and 3.77 m s<sup>-1</sup> were investigated on a 5 L batch scale. Stirrer speeds were increased in order to decrease oil droplet size and hence increase lipid utilisation. Increasing the tip speed from 1.88 to 2.83 m s<sup>-1</sup> extended hyphal development. At 3.77 m s<sup>-1</sup> fragmentation occurred. Raising tip speed decreased specific lipase activity and had no significant effect on lipid utilisation and residual oil levels. Research concludes that the microscale of turbulence could not be reduced to a small enough diameter to increase oil drop breakage at power input levels used in pilot plant and production scale vessels.

7. The addition of the surfactant, SDS, to the process medium increased lipid utilisation and enhanced clavulanic acid titres. Residual oil levels in the SDS fermentations were due to additional antifoam requirements. The surfactant had increased rapeseed oil dispersion, reducing its natural antifoam properties. Gas chromatography of the fermentation broth revealed a decrease in residual oil levels from 30% to 0%. Increased dispersion of the rapeseed oil was noted through off-line capacitance measurements. The benefits of raised product titres and reduced residual oil must be balanced against additional downstream processing costs through additional antifoam requirements and surfactant removal. Although research into the use of surfactants, with respect to oil utilisation, has proved inconclusive the theories investigated may be developed further for use on production scale.

## **7.2. Recommendations**

These experiments form the basis of initial studies into the factors involved in lipid utilisation in 5 L batch fermentations of *Streptomyces clavuligerus* using complex process media. The results have shown that increasing dispersion of the oil substrate in the media is the key to enhancing productivity through increased lipid utilisation. The

addition of a surfactant to the media, although proved to increase productivity, has the additional costs of downstream surfactant removal and increase antifoam concentration. SDS may have led to a decrease in oil utilisation through interference displacing the lipase enzyme. Further studies may include an investigation into the use of a fully metabolisable emulsifier, such as Disponil (supplied by Henkel) or the use of prehomogenised media. Primitive studies were carried out into the homogenisation of media presterilisation and initial observations showed an increase in rapeseed oil dispersion.

As the dispersion of the oil is a key parameter in utilisation, an area of research that might be of interest is *in situ* particle size analysis. Off-line particle size analysis has been carried out using oil-water mixtures (Cavanagh, 1996) but on-line analysis would give a better representation of the oil droplet size within the fermenter environment and the effect of surfactants, emulsifiers and media homogenisation. Particle size analysis could then be correlated with oil utilisation and a model formed which could be applied on a production scale.

Lipase activity was concluded not to be a limiting factor in the utilisation of rapeseed oil. However, to use the lipase activity effectively in the fermentation containing complex media, a fundamental understanding of the role of substrates, products and the site of catalysis are required. The size of the oil-water interface is of importance when studying lipase activities. An understanding of the effects of the interfacial area could also be correlated to the droplet size-oil utilisation model.

Concentration of rapeseed oil may affect lipid dispersion and hence the rate of utilisation. Methods of feeding oil may keep the concentration of oil at a low enough level to become dispersed yet high enough for cell growth and secondary metabolite production. Different methods of feeding, nozzle design and placement may also enhance droplet dispersion and therefore reduce residual oil levels and so increase productivity.

Shake flask experiments using the individual triglyceride and methyl ester constituents of rapeseed oil showed little difference in growth rate but scale down may have affected productivity. Although expensive, larger scale fermentations are necessary to confirm

the effects of the individual components of rapeseed oil on productivity. Individual triglycerides could not be used on scale up due to cost but different oils may be selected depending on their percentage triglyceride content.

The lipase activity detected in *Streptomyces clavuligerus* has been reported to be associated with the mycelial membrane. Although lipid utilisation seems relatively unrelated to levels of detected lipase activities, an area of research that may be of interest is the cloning of an additional exogenous lipase. This may be possible as streptomyces genetics evolve. All lipases in streptomyces studied within the research group (Large *et al.*, 1999) have been shown to be associated with the mycelial membrane, meaning the interface must come to the organism or the organism to the interface. Additional exogenous lipase activity may increase lipid utilisation and operate in conjunction with the already present cell associated lipase activity to enhance fatty acid input to the  $\beta$ -oxidation cycle.

Experiments involving addition of SDS to the media revealed that it may not only be dispersion of the oil which enhances productivity, but increased titres may also be due to additional sulphate and/or sodium. Another line of fermentation based research would involve enhancing product titres, and maybe lipid utilisation, through investigation of the effects of media components such as sulphates, phosphates and sodium. Iron dependent enzymes are present in the clavulanic acid pathway and cluster analysis in media development, as described by Ives and Bushell (1998), and this may reveal other control mechanism which could increase product titres.

## 8. APPENDICES

### Appendix I. Sample microscale of turbulence calculation:

Ungassed power ( $P_{ug}$  - W) is proportional to the power number of the impeller used ( $P_o$ ), density of the media (as water,  $1000 \text{ Kg m}^{-3}$ ), the impeller speed ( $N_i$  - rps) and diameter ( $d_i$  - m):

$$P_{ug} = P_o \rho N_i^3 d_i^5 \quad (8.1.)$$

A process containing 3 Rushton turbines of 0.06 m diameter at 600 rpm ( $10 \text{ rps}^{-1}$ ):

$$(3 \times 4.5) \times 1000 \times 10^3 \times 0.06^5 = \mathbf{10.5 \text{ W}}$$

Gassed power in to the vessel is equal to the ungassed power multiplied by the impeller speed and diameter divided by total gas flow ( $Q$  -  $\text{m}^3 \text{ s}^{-1}$ ).

$$P_g = 0.72[(P_{ug}^2 N_i d_i^3)/Q^{0.56}]^{0.45} \quad (8.2.)$$

For the above fermentation with a gas flow rate of  $8.3 \times 10^{-5} \text{ m}^3 \text{ s}^{-1}$   $P_g$  :

$$0.72[(4.22^2 \times 10 \times 0.06^3)/(8.33 \times 10^{-5})^{0.56}]^{0.45} = \mathbf{4.03 \text{ W}}$$

Power per unit volume is calculated using the gassed power over vessel volume,  $P_g/V$ :

$$4.03/0.005 = \mathbf{805.10 \text{ W m}^3}$$

To calculate the microscale of turbulence -  $\lambda$  :

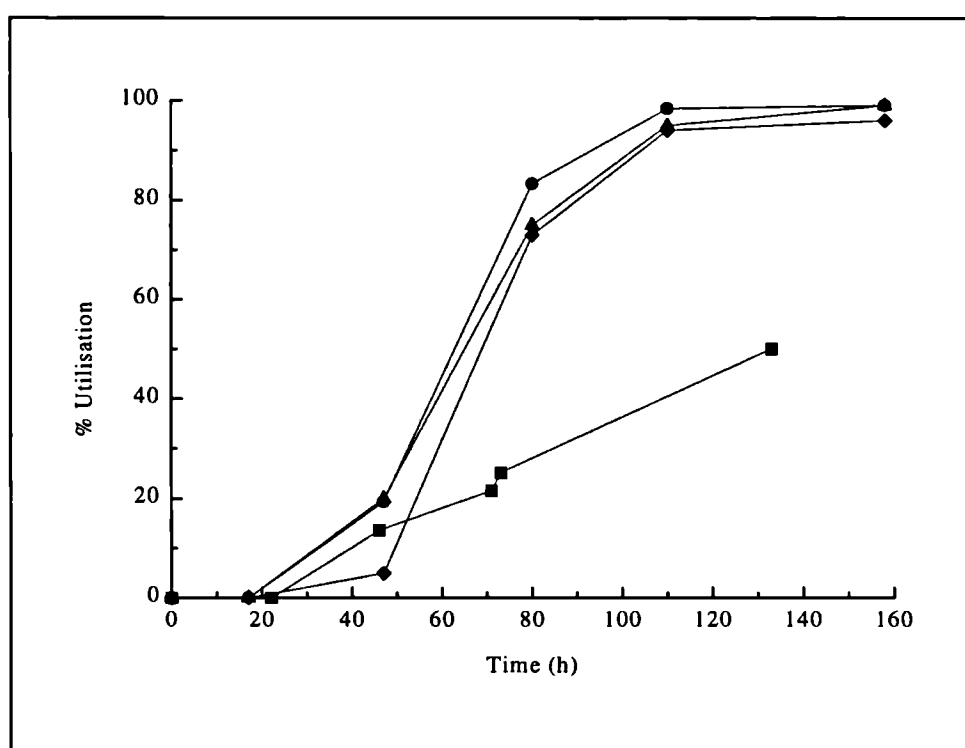
$$\lambda = (\nu^3 / \epsilon)^{0.25} \quad (8.3.)$$

where  $\nu$  is equal to the kinematic viscosity of the fluid and  $\epsilon$  is the local rate of turbulent energy dissipation per unit mass of liquid ( $\text{W Kg}^{-1}$ ). Calculations assumed equal energy distribution throughout the bioreactor. For a fermentation with an initial viscosity  $0.007 \text{ Kg ms}^{-1}$ :

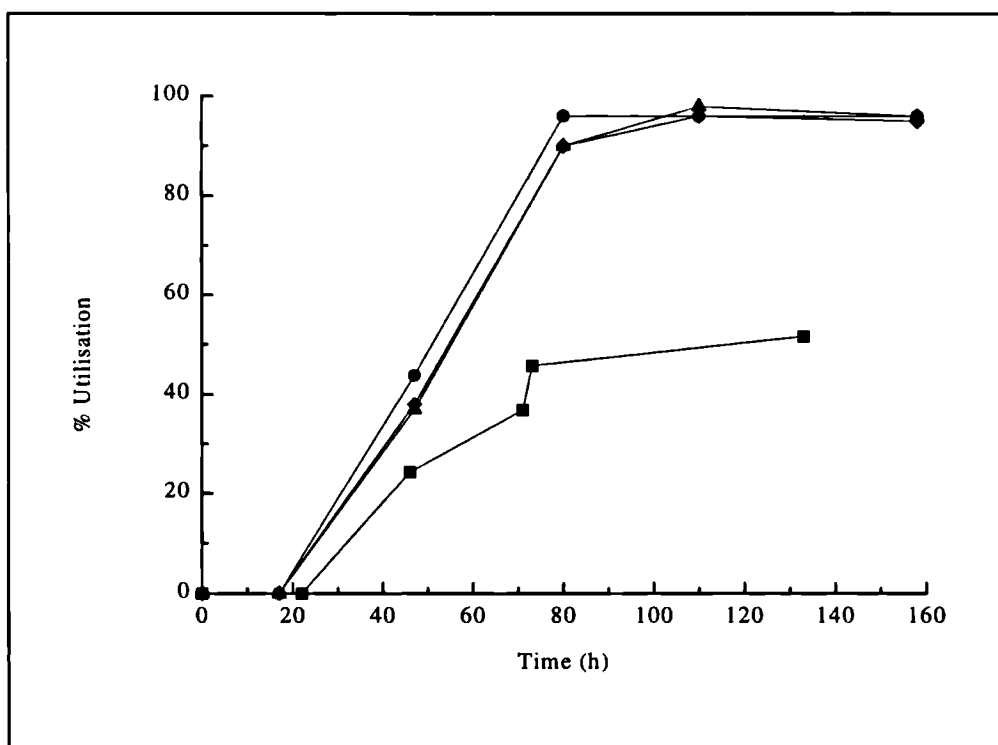
$$[(7.0 \times 10^{-6})^3 / (805.1 / 1000)]^{0.25} = \mathbf{143 \mu\text{m}}$$

## Appendix II. Effect of SDS on individual fatty acid utilisation

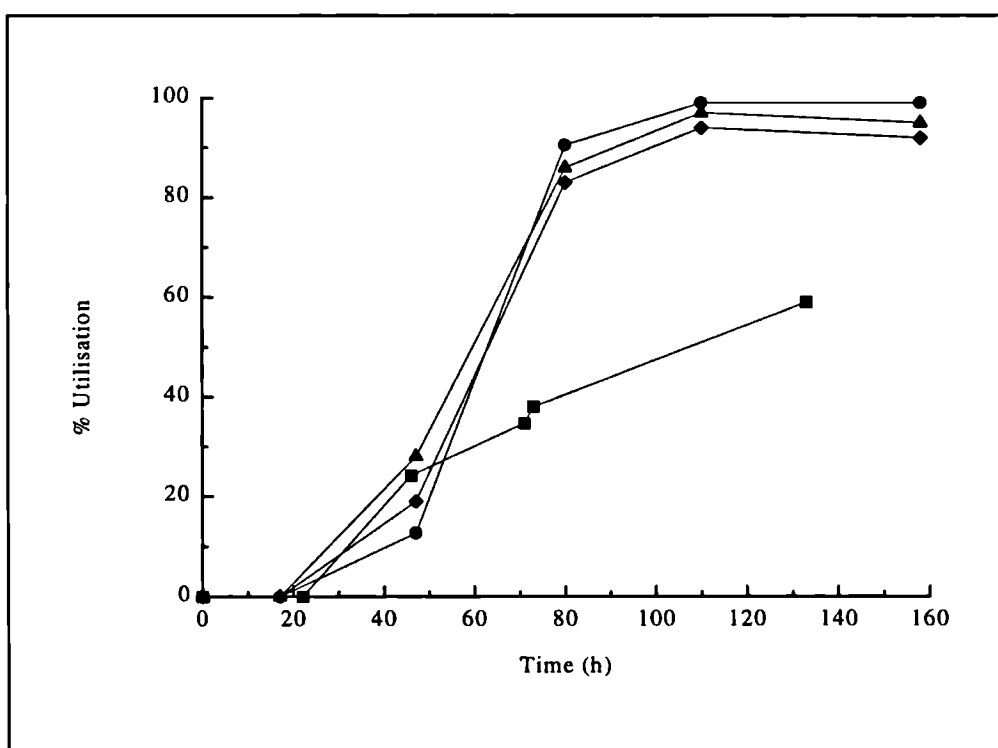
The effect of different concentrations of SDS on fatty acid utilisation in the 5 L batch fermentation of *S. clavuligerus* on complex media were shown in Figure 6.11. To clarify the effect of increasing concentrations of SDS on fatty acid utilisation profiles were created for each individual fatty acid showing percentage utilisation as a function of SDS concentration. These data show that it is the presence of SDS and not the concentration that affects the percentage utilisation of specific fatty acids.



**Figure 8.1. Percentage palmitic acid utilisation at 0% ( ■ ), 0.05% ( ◆ ), 0.075% ( ▲ ) and 0.1% ( ● ) SDS.**

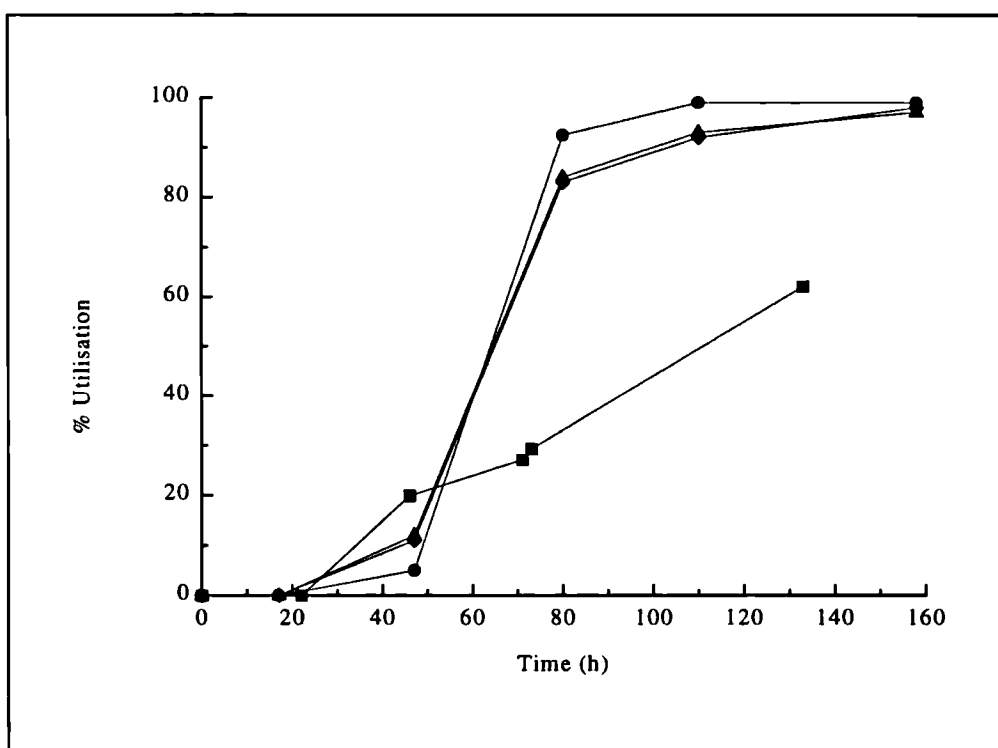


**Figure 8.2. Percentage stearic acid utilisation at 0% (■), 0.05% (◆), 0.075% (▲) and 0.1% (●) SDS.**

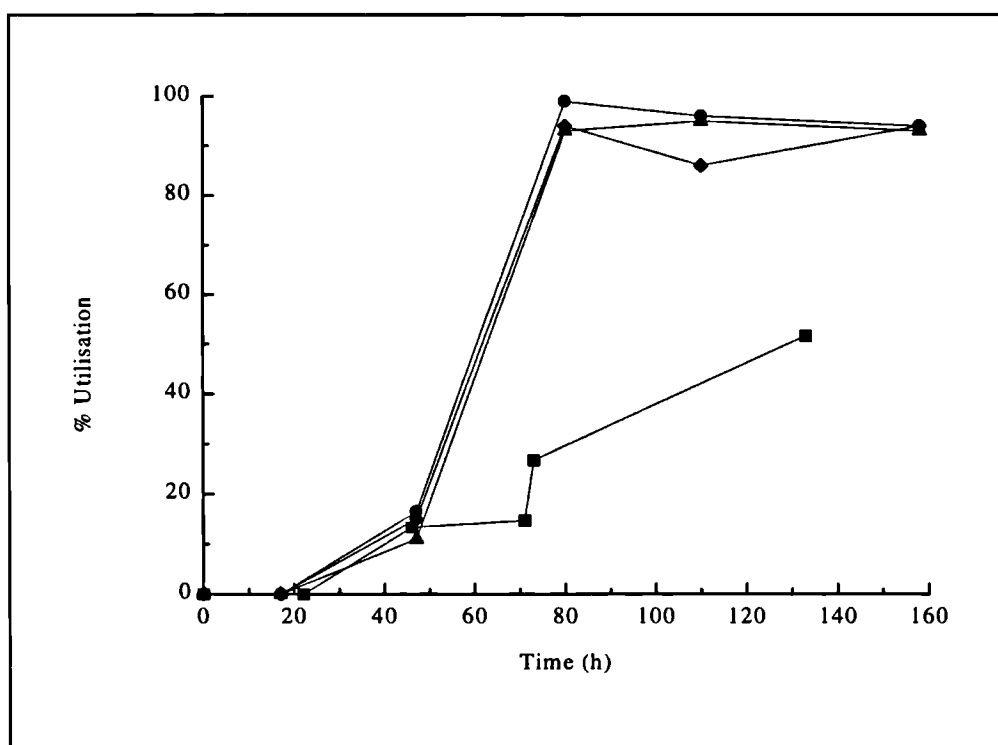


**Figure 8.3. Percentage oleic acid utilisation at 0% (■), 0.05% (◆), 0.075% (▲) and 0.1% (●) SDS.**





**Figure 8.4. Percentage linoleic acid utilisation at 0% (■), 0.05% (◆), 0.075% (▲) and 0.1% (●) SDS.**



**Figure 8.5. Percentage linolenic acid utilisation at 0% (■), 0.05% (◆), 0.075% (▲) and 0.1% (●) SDS.**

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